

## LOW AND HIGH RESPONDERS AFTER POLYPROPYLENE MESH IMPLANTATION FOR INGUINAL HERNIOPLASTY

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### ABSTRACT

The aim of the study was to evaluate individual inflammatory response after inguinal hernioplasty by measuring baseline and post-operative serum IL-6 response to surgery and quantifying the variability of the serum response in a homogeneous group of patients. Patients enrolled in the study underwent inguinal hernioplasty. Blood samples were used to analyze serum IL-6, TNF-alpha, LOOH and GSH levels. To identify high and low IL-6 responders, patients were divided into 2 groups according median values of the peak levels of IL-6. Mean levels of the cytokine were comparable at baseline but reached peak at 6 h and persisted elevated at following hours among high responders whereas remained significantly lower over time among low responders. Patients identified as low responders showed not significant changes in serum IL-6 levels over the post-operative period. In contrast, high responders demonstrated a significant response reaching the peak level of IL-6 after 6 hours post operation.

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### 1. Introduction

Inflammation is a phenomenon that occurs throughout perturbations of homeostasis, for instance after surgical proceedings, and may be assigned to either physiological or pathological processes (1). Mediators of inflammatory response, such as cytokines and chemokines, trigger production of reactive oxygen species (ROS) that are involved in induction of host defense genes but also may cause damage of cellular components (2). Although innate immunity is crucial for adequate host responses to infection and is implicated in wound healing (3) and angiogenesis (4), local and systemic inflammatory responses during and after surgery are involved in the development of postoperative complications and in relatively fast general restitution (5). Furthermore, presence in blood stream of pro-inflammatory cytokines, such as IL-6 and TNF-alpha has been linked to pathological pain (6) and hypergelsia (7).

Several studies have demonstrated that basal level of pro-inflammatory cytokines (8) and inflammatory response may differ between individuals due to physiological factors acting on different aspects of cellular and systemic physiology (i.e., gender, age) and multiple genetic (9-12). Since both inflammation and oxidative stress may lead to post-surgery complications, the evaluation of inflammatory response has been the focus of recent studies as possible predictors of outcome. Studies on hernioplasty reported significantly increased level in serum IL-6 concentration after incidence (13-15). However all studies on hernioplasty took into consideration different surgical procedures by mean of the use of different technics or operative materials, but to the best of our knowledge, yet no evidence determining inter-individual variability in IL-6 response to hernioplasty has been presented. Moreover, it is of primary importance to test whether plasma cytokines, such as IL-6, are reliable markers of inflammatory response (16) or differences between individuals should be taken into account. The aim of the study was to evaluate the different individual inflammatory response after inguinal hernioplasty by

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measuring baseline and post-operative serum IL-6 response to surgery and quantifying the variability of the serum response in a homogeneous group of patients. Accordingly, we also evaluated TNF-alpha levels and the oxidative stress response by measuring baseline and post-operative LOOH and GSH and characterized modulator factors of this response.

## 2. Material and Methods

### Study population

Between March 2010 and December 2011, patients were prospectively recruited when referred to the hernia service of the General Surgery and Week Surgery Unit of the University Hospital of Catania. The patients were recruited in the context of a randomized controlled trial (registration number at www.clintrials.gov NCT01090284) aimed to evaluate possible differences among responses between two types of meshes ("light" and "heavy"). Patients' inclusion criteria were i) primary inguinal hernia, ii) age between 18 and 90 years, iii) no previous operation with implantation of a prosthetic mesh. Exclusion criteria were i) presence of diabetes, cirrhosis, or any chronic inflammatory disease, ii) current corticosteroid therapy, and iii) current immunosuppressive therapies (neoplastic patients). A total of 62 patients were recruited. However, one patient was excluded due to previous operations with mesh implants, one patient was affected by immunological disorders (multiple sclerosis), and one patient did not return after the operation to complete the requested protocol for blood analyses. The final number of patients examined was 59.

After confirmation of inguinal hernia diagnosis by physical examination and indication for surgery, informed consent was obtained from the patient by means of a standardized form. The study was carried out according to the declaration of Helsinki and the study protocol was approved by the ethic committee of the University of Catania.

### Surgical technique

All the patients underwent an open local anesthesia prosthetic inguinal hernia repair as gold standard technique (17), a variant of Trabucco's repair with the apposition of one or more plugs (18, 19). Two types of meshes were used, differing by weight (g/m<sup>2</sup>) (content of polypropylene), but equal for pore size and texture (HERTRA, Herniameshs.r.l. Chivasso, Turin, Italy). Surgical interventions were performed by the same operator.

### Blood sample analysis

For each patient, blood samples were obtained to determine preoperative basal levels of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), reduced glutathione (GSH) and lipid hydroperoxides (LOOH), 6 hours, 72 hours (3rd day) and 288 hours (12th day) after operation.

All samples were frozen at -80 °C until measurements. Plasmatic levels of LOOH were evaluated by assessing the oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup> in the presence of xylenol orange at  $\lambda = 560$  nm.

The assay mixture contained, in a total volume of 1 ml/100  $\mu$ l of plasma, 100  $\mu$ M xylenol orange, 250  $\mu$ M ammonium ferrous sulfate, 90% methanol, 4 mM butylatedhydroxytoluene, and 25 mM H<sub>2</sub>SO<sub>4</sub>. After a 30-minute incubation at 27 °C, the absorbance was measured using a U2000 Hitachi spectrophotometer (20). Calibration was done using 0.2–20  $\mu$ M hydrogen peroxide. Experiments were performed in duplicate. Plasmatic levels of GSH were measured in 200  $\mu$ l of plasma, using a spectrophotometric assay based on the reaction of thiol groups with 2,2-dithio-bis-nitrobenzoic acid (DTNB) at  $\lambda = 412$  nm ( $\epsilon M = 13,600$  M<sup>-1</sup>cm<sup>-1</sup>, where  $\epsilon M$  is a wavelength-dependent molar absorptivity coefficient) (21-23). Experiments were performed in duplicate.

Each cytokine was determined by commercially available ELISA kit (Ebioscience, Prodotti Gianni, Milan, Italy) according to manufacturer's protocol (24). According to previous studies (8), the intra-individual variability was tested to evaluate whether variability in IL-6 could be attributable to laboratory test. The within-coefficient of variation (CV<sub>w</sub> = Sw/mean of each measurement) was calculated, where Sw is the square root of the estimated intra-individual variance and a desired acceptable value lower than 20%. IL-6 CV<sub>w</sub> was 18,7%, thus satisfying the threshold proposed.

### Statistical analysis

Continuous variables are presented as mean  $\pm$  standard errors (SE). Normality of variables' distribution was tested by Kolmogorov-Smirnov test. Because data were not normally distributed, non-parametric tests were used. To identify phenotypical high and low responders to IL-6, patients were divided into 2 groups according to median values of the peak levels of IL-6. For both groups, TNF-alpha, GSH and LOOH mean response level were evaluated. Mann-Whitney U-test was used to determine potential differences between groups and Friedman's ANOVA was used to determine whether there were any significant differences over time for individual cytokine. If a significant difference was present, a Dunn's post-hoc test was used to locate the difference. The area under the response curve (AUC) was calculated for the day of intervention according to the trapezoid method and compared between groups by Mann-Whitney U-test. The Spearman rank correlation was performed to identify potential relationships of serum inflammatory markers levels at baseline and AUCs with gender, age, BMI, operative time, number of plugs, and type of mesh.

All statistical tests were two-tailed and a p-value  $\leq 0.05$  was considered significant. Data were entered into Microsoft Excel for Windows (Microsoft Corporation, Redmond, WA). Statistical analysis was performed using SPSS for Windows release 20.0 (SPSS Inc., Chicago, IL, USA).

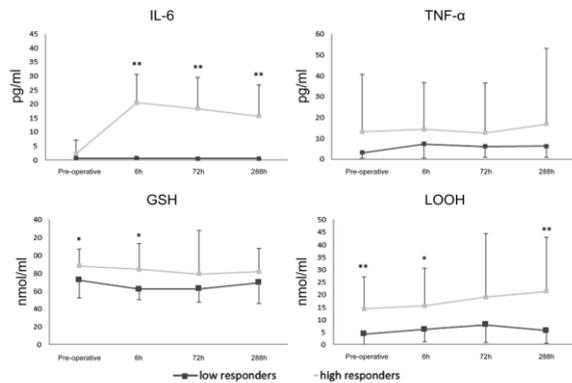
## 3. Results

Background characteristics according to low and high responders are presented in Table 1. The analysis showed no significant differences between the two groups, suggesting homogeneity of the study sample.

	Low responders	High responders	P
Age	60.6 $\pm$ 13.6	59.0 $\pm$ 10.7	0.620
Mesh type			0.438
- Light	16 (53.3)	12 (41.4)	
- Heavy	14 (46.7)	17 (58.6)	
No of plugs	1.5 $\pm$ 0.8	1.7 $\pm$ 0.9	0.414
Time of operation	105.2 $\pm$ 32.5	111.6 $\pm$ 34.9	0.468
BMI	25.2 $\pm$ 3.1	26.2 $\pm$ 3.9	0.297

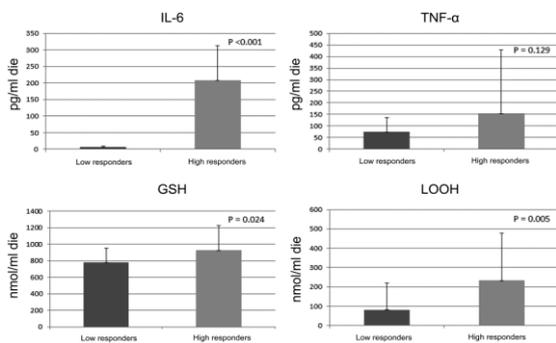
Table 1 - Baseline characteristic of high and low responders.

The mean peak levels of IL-6 were reached at 6 h (Figure 1). Mean levels of the cytokine were comparable at baseline but reached a peak at 6 h and persisted elevated at following hours among high responders whereas remained significantly lower over time among low responders (Figure 1). No differences between high and low responders were found for TNF-alpha. Among oxidative stress markers, LOOH levels were significantly lower among low responders compared with the high except at 288 hours when the peak was reached in the former (Figure 1). Similarly, also GSH levels significantly differed at baseline and early hours after surgery, but the difference was no longer significant at later follow up times (Figure 1). When analyzing variations over time, only IL-6 significantly differed at follow-up visits from baseline whereas peak levels of TNF-alpha and LOOH among high responders seemed to be reached later, despite still not significantly higher than baseline levels (Figure 1). Overall, changes among low responders had similar non-significant pattern over time for all inflammatory and oxidative makers, despite less pronounced for IL-6 (Figure 1).



**Figure 1** - Serum IL-6, TNF-alpha, GSH and LOOH responses to inguinal hernioplasty in high and low responders. Statistically significant differences between the two groups are highlighted (\* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). Values are presented as means  $\pm$  SD

AUC significantly differed between high and low responders for IL-6, LOOH, and GSH, but not for TNF-alpha (Figure 2). Among the variables potentially associated with baseline mean levels and AUCs, only BMI was significantly directly correlated with IL-6 AUC among high responders ( $R = 0.406$ ,  $P = 0.029$ ), but not in low-responder patients (Table 2).



**Figure 2** - Mean AUC value for serum IL-6, TNF-alpha, GSH and LOOH concentration in high and low responders to inguinal hernioplasty. Statistically significant differences between the two groups are highlighted. Values are presented as means  $\pm$  SD

	LOOH	LOOH AUC	GSH	GSH AUC	IL-6	IL-6 AUC	TNF-alpha	TNF-alpha AUC
High responders								
Mesh type	-0.025	0.117	-0.042	-0.343	-0.083	-0.167	0.158	0.284
Age	0.096	0.222	-0.189	-0.189	0.052	-0.2	-0.376*	0.026
No of plugs	-0.02	-0.134	0.144	0.135	-0.15	0.015	-0.21	0.047
Time of operation	-0.156	0.04	0.093	-0.043	-0.311	0.194	-0.279	-0.094
BMI	0.1	0.125	0.124	-0.079	-0.224	-0.095	-0.039	-0.013
Low responders								
Mesh type	0.046	0.124	-0.255	-0.147	-0.224	-0.081	-0.207	0.008
Age	-0.012	-0.013	0.053	0	0.2	0.066	0.123	-0.238
No of plugs	-0.354	-0.132	-0.03	-0.007	0.3	0.315	-0.202	-0.151
Time of operation	-0.224	-0.13	-0.004	-0.113	0.239	0.151	0.085	-0.082
BMI	-0.128	-0.054	0.296	0.440*	0.253	0.406*	-0.256	0.009

\* Correlation is significant at the 0.05 level (2-tailed).

**Table 2** - Correlation of potential variables with mean AUC levels.

## 4. Discussion

The aim of this study was to measure the degree of inter-individual variability in the acute immune response after hernioplasty by determining high and low serum IL-6 responders. Our results showed that mean pre-operative levels of serum IL-6 were similar among patients and comparable to the physiological concentrations. After hernioplasty, patients identified as low responders showed not significant changes in serum IL-6 levels over the post-operative period. In contrast, high responders demonstrated a significant response reaching the peak level of IL-6 after 6 hours post operation.

Accordingly, also TNF-alpha and oxidative stress markers levels were consistent with IL-6 response, despite differences between low- and high-responder patients were less remarkable. We reported lack of significant difference in baseline serum IL-6 concentrations and scarce inter-individual variability. A reason for such finding may depend on the fact that an exclusion criterion for our trial was presence of any acute or chronic disease. Thus, selected patients were most likely to be comparable to healthy individuals, which have been demonstrated to have consistent IL-6 concentration irrespectively of age, gender (8), or genetic pool (16). In contrast, the variability of serum baseline TNF-alpha concentration among patients was higher (data not shown), in line with previously reported findings of a study demonstrating the role of TNF-alpha gene polymorphism, rather than age or gender, in determining such variability (17).

In the present study, we also measured concentration of oxidative stress markers. Our results showed that GSH concentration decreased over the post-operative period. As the role of GSH is to prevent important cellular components against damage caused by reactive oxygen species the decline of GSH concentration while oxidative stress is a physiological mechanism. In contrast, LOOH levels increased, most likely due lipid oxidation following the production of ROS(18). However, LOOH levels increase was delayed compared with IL-6 response.

This dynamic may be explained by physiological mechanisms during acute inflammation. In fact, the initial infiltration of neutrophils is counteracted by IL-6 release in the blood stream that switches recruitment from neutrophils to monocytes and subsequently promotes differentiation

of monocytes into macrophages (19). The replacement of neutrophils by monocytes and T-cells after 24–48 hours prevents increased tissue damage from the accumulation of neutrophil-secreted proteases and ROS at the site of inflammation (19).

As a result of this mechanism, the effect of ROS leading to increase of LOOH is delayed.

Even though there were no differences in baseline concentration, evidence of a remarkable variability between patients in the serum IL-6 response to hernioplasty was found. Taking into account that the surgical procedure was performed in the most reproducible way and physiological differences in the local anatomy and in the anatomical characteristics of the disease influenced duration time of the intervention and number of applied plugs, none of the variables related with the surgical procedure that may possibly influence the inflammatory response (i.e., duration of intervention) was not found to be determinant for low and high serum IL-6 response.

In contrast, the analysis of AUC revealed a significant inverse correlation between the response degree of TNF- $\alpha$  with patients' age. It is well known that immune system changes during the aging process. The ability of the adaptive immune system to cope with age-associated changes is limited, primarily due to the decline in production of naive lymphocytes in the bone marrow and thymus and the increase of incompetent memory lymphocytes (13). However, patients classified as low-responders reported mostly complete lack of response, suggesting that also other mechanisms may account for such condition.

One study showed implication of genotype in the degree of response (25) by demonstrating that IL-6 peak production was correlated with IL-6 promoter polymorphism.

Curiously, BMI was correlated with the level of response (i.e., IL-6 AUC), suggesting that body mass index, or rather specifically adipose tissue may influence inflammatory system (26–28). However, such correlation was not confirmed among high responders, and further studies are needed to clarify such potential association. A limitation of this study to be taken into account when considering our results is the limited number of patients enrolled.

Although a small sample size may lead to limitation in variables explored due to lack of statistical power, patients enrolled in this study have been selected in the context of defined inclusion and exclusion criteria, therefore characterized by homogenous health and medical background that should attenuate the effects of potential confounders on immune system.

However, further larger studies are needed to expand the number of investigated markers and profile gene expression of those genes that have been reported to be implicated in regulation of inflammatory response in individuals.

In conclusions, our study supports the use of IL-6 as good marker of inflammation as variation in baseline levels among healthy individuals is scarce. However, IL-6 peak level and AUC were found to be the most informative parameters for quantifying the amount and dynamics of the serum IL-6 response, as some patients may experience lack of significant response (low-responders) and need to be addressed at individual level before proceeding at group level considerations (29).

A better understanding of inter-individual variation in acute immune response mechanisms will facilitate the discovery of novel molecular markers of poor post-surgery outcomes and will contribute to establishment of the most efficient treatment strategy for patient retrieval.

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