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Original research article

HLA-G and anti-HCV in patients on the waiting list for kidney transplantation

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ABSTRACT

Purpose: Human leukocyte antigen (HLA)-G is a non-classic major histocompatibility complex HLA class I molecule. HLA-G may have tolerogenic properties which are linked to epigenetic-sensitive pathways. There is a correlation of sHLA-G levels and graft acceptance in transplantation studies. There are previous data on correlation of sHLA-G with graft rejection as well as with viral infections such as hepatitis C virus (HCV) in kidney transplanted patients. Here, we report the sHLA-G expression in patients on the waiting list for kidney transplantation, with and without anti-HCV compared to a control group.

Methods: Serum of 67 patients on the waiting list for kidney transplantation (n = 43 with anti-HCV and n = 24 without anti-HCV) was analyzed. Among these patients, n = 39 were on the waiting list for the first transplantation, while n = 28 were patients who returned in the list. The control group included n = 23 blood donors with anti-HCV (n = 13) and without anti-HCV (n = 10).

Results: The expression of sHLA-G was significantly lower in the control group (39.6 ± 34.1 U/ml) compared to both - patients on the waiting list for the first transplantation (62.5 ± 42.4 U/ml, $p = 0.031$) and patients who returned in the list (76.7 ± 53.9 U/ml, $p = 0.006$). No significant differences were observed in all anti-HCV positive groups. A positive linear correlation between sHLA-G and TNF-α, and patient age was observed.

Conclusions: Serum sHLA-G values were significantly increased in both - patients on the waiting list for the first transplantation and patients who returned in the list, as compared to control group. Our findings confirm the key tolerogenic role of sHLA-G levels as epigenetic-related marker for measuring the state of kidney allograft acceptance.

1. Introduction

The human leukocyte antigen-G (HLA-G) got much attention due to its multiple functions in the immune system as well as into epigenetic platform [1–6]. It plays a fundamental role in inducing tolerance by its immunosuppressive effects on all types of immune cells [7,8].

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HLA-G is a non-classic major histocompatibility complex class Ib antigen characterized by a low allelic polymorphism. Its expression is regulated through epigenetic mechanisms (alternative mRNA splicing), which produce different isoforms - four of them are membrane-bound (HLA-G1-G4), and three soluble (HLA-G5-G7); in addition, HLA-G1 isoform can produce a soluble form called sHLA-G1 derived from the membrane proteolytic shedding [9].

HLA-G expression was initially described in the maternal-fetal interface; it protects the fetus from destruction by its mother's immune system [10,11]. Afterwards, it was shown to contribute to tumor escape. It was also shown that HLA-G was involved in the protection of the transplanted tissues *via* the inhibition of all immune effectors that mediate graft rejection [12].

Detecting it may also serve as a clinical marker in predicting viral infections [13–16]. Hepatitis C virus (HCV) infection is a worldwide public health problem. Current dogma suggests that immunity to infection is controlled by T helper type (Th1 and Th2)-type immunity; Th1 and Th2 cell subsets are crucial in determining cytokine release [17,18]. In addition, several studies observed a high plasma soluble HLA-G (sHLA-G) levels in patients with chronic HCV infections; therefore, HLA-G protein could be considered a potential candidate involved in modulating susceptibility to HCV persistence and chronicity [16,19–21]. It was demonstrated that HLA-G acts on all immune response cells [22]; indeed, it was proved that HLA-G molecules inhibit cytotoxic activity of T cells, natural killer cell lysis, alloproliferative response, maturation of dendritic cells and can also be involved in generating regulatory cells [22].

Studies showed that high expression of sHLA-G on monocytes was associated with kidney allograft acceptance and that the presence of sHLA-G dimers linked to the lower levels of pro-inflammatory cytokines plays a potential role in controlling the inflammatory state [23,24]. HLA-G molecule has a high potential function to modulate the immune response towards the improvement of kidney graft survival in pre- and post-transplantation [22,25–28]. The up-regulation of sHLA-G expression in kidney transplant recipients without rejection compared to those with rejection was confirmed [29,30].

The aim of this study was to evaluate the expression of sHLA-G levels both in patients on the waiting list for kidney transplantation and in patients who returned in the list after the first transplantation by comparing them with blood donors as a control group. In addition, we investigated the correlation between sHLA-G levels and the presence of antibodies against HCV (anti-HCV).

2. Materials and methods

2.1. Study population

From June 2014 to May 2015, a total of 67 patients on the waiting list for kidney transplantation (53 men and 14 women) with a mean age of 56.2 ± 9.8 (range 26–77 years) with anti-HCV (n = 43) and without anti-HCV (n = 24) were included in this study. Among these patients, n = 39 were on the waiting list for the first transplantation (n = 25 with anti-HCV and n = 14 without anti-HCV), while n = 28 patients returned in the list after the first transplantation (n = 18 with anti-HCV and n = 10 without anti-HCV). The control group included n = 23 blood donors (17 men and 6 women) with mean age of 50.5 ± 9.3 (range from 32 to 65 years) with anti-HCV (n = 13) and without anti-HCV (n = 10). Serum samples from the subjects were preserved at −70 °C following centrifugation until assayed. A written consent was obtained from all patients. Both study groups and the control group did not show any HCV infection. In absence of major comorbidity contraindication renal transplantation, no description of clinic outcomes of patients and control group was reported.

2.2. Serum screening for HCV

Serum samples were tested for anti-HCV by chemiluminescent immunoassays (CMIA) on the ARCHITECT platform (Abbott Diagnostics, Wiesbaden, Germany) followed by HCV specific immunoblot assays as confirmatory testing (INNO-LIA, Innogenetics, Ghent, Belgium). Furthermore, all sera were also screened by NAT for HCV-RNA with the TaqScreen method on the Cobas s201 system (Roche Molecular Systems, Branchburg, NJ, USA): the assay was performed on mini pools of six samples each and has a nominal sensitivity of < 20 IU/mL. Each assay was performed in a single run for each specimen, and was carried out according to the respective manufacturer's instructions [31,32].

2.3. sHLA-G levels

Serum sHLA-G expression was determined with the sHLAG-specific ELISA kit (sHLA-G kit; BioVendor, Czech Republic) which measures HLA-G1 and HLA-G5. Each sample (100 µl) was measured in triplicate. The optical densities were measured at 450 nm (TECAN Infinite M200 station). Finally, sHLA-G concentrations (U/mL) in the samples were calculated using the calibration curve constructed by plotting the ODs against concentrations of calibrators provided by the manufacturer. The detection limit of ELISA kit was 0.6 U/mL. Details of the performance were according to the manufacturer's instruction.

2.4. Cytokine analysis

TNF-α and IL-10 serum levels were measured using enzyme-linked immunosorbent assay (ELISA) (pg/ml) (ELISA, R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. Each sample (100 µl) was measured in triplicate. The minimum detectable dose (MDD) of TNF-α ranged from 0.5 to 5.5 pg/mL and the mean MDD was 1.6 pg/mL. The MDD of human IL-10 is typically less than 3.9 pg/mL. The values were read at 450 nm in an ELISA reader, and TNF-α and IL-10 concentrations were calculated from specific calibration curves prepared with known standard solutions.

2.5. Statistical analysis

All statistical analyses were carried out using SPSS 13.0. Box-plot of sHLA-G was organized by control group, patients on the waiting list for the first transplantation, and patients who returned in the list after the first transplantation. Data are expressed as median ± SD. Mean differences of sHLA-G between groups were estimated using independent sample *t*-test. Normal distribution of scalar parameters was assessed by Kolmogorov-Smirnov test. All parameters were normally distributed. Differences between anti-HCV negative and positive patients in sHLA-G were estimated using independent sample *t*-test. Pearson correlation was used to evaluate the correlation between sHLA-G and TNF-α, IL-10 and age in all patients with absence or presence of anti-HCV. A value of $p < 0.05$ was considered statistically significant.

2.6. Ethical approval

All procedures performed in this study were in accordance with the ethical standards of our institutional research and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all patients involved in this study.

Although our work was conducted under conformity of Declaration of Helsinki and no additional blood samples were taken, for all activities including the waiting list for organ transplantation and/or blood donation the approved Number of Ethic Committee, University of Campania "Luigi Vanvitelli" is Number: 295.

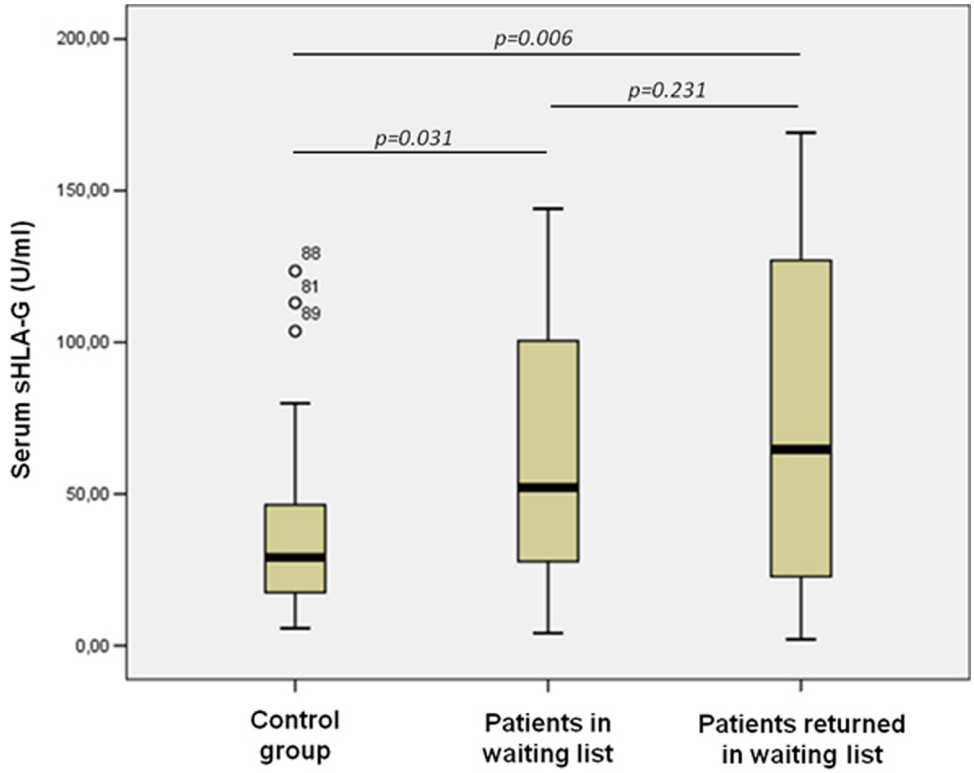

Fig. 1. Distribution and comparison of serum soluble human leukocytes antigen-G among the studied groups. The bar in the box represents the median. Comparison of sHLA-G levels among the groups was performed using *t*-test.

3. Results

In Fig. 1 we present the serum sHLA-G levels with Box-plot organized by control group, patients on the waiting list for the first transplantation, and patients who returned in the list after the first transplantation. The expression of sHLA-G was significantly lower in the control group (39.6 ± 34.1 U/ml, n = 23) when compared to both, patients on the waiting list for the first transplantation (62.5 ± 42.4 U/ml, $p = 0.031$, n = 39) and patients who returned in the list after the first transplantation (76.7 ± 53.9 U/ml, $p = 0.006$, n = 28). No significant difference was found in sHLA-G expression between the patients who returned in the list after the first transplantation and those on the waiting list for the first transplantation.

In Fig. 2 we report the serum sHLA-G levels in the three groups in relation to the presence of anti-HCV. In particular, in the control group the difference of sHLA-G between blood donors without and with anti-HCV was not significant (28.5 ± 21.3 *vs* 48.1 ± 40.2, $p = 0.178$). Similarly, in patients on the waiting list for the first transplantation, the difference of sHLA-G between patients without and with anti-HCV was not significant (52.8 ± 42.5 *vs* 67.8 ± 42.2, $p = 0.292$). In relation to the absence or presence of anti-HCV, the difference of sHLA-G resulted not significant also in the group of patients who returned on the list (93.6 ± 49.9 *vs* 67.4 ± 55.0, $p = 0.225$). Significant differences were observed in the control group with respect to patients who returned on

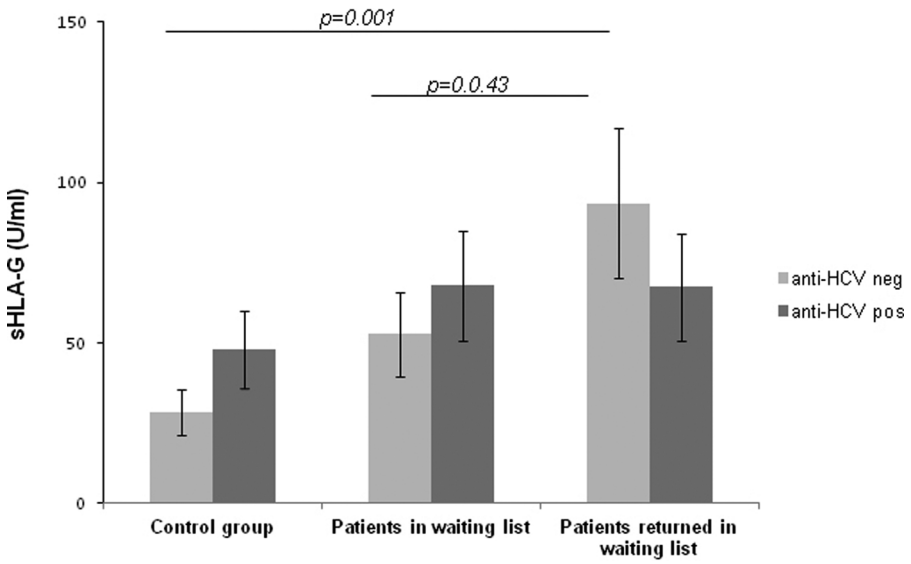

Fig. 2. Distribution and comparison of serum soluble human leukocytes antigen-G among the studied groups in relation to the absence or presence of anti-HCV. Control group anti-HCV-neg *vs* patients on the waiting list anti-HCV-neg, $p = 0.112$; control group anti-HCV-neg *vs* patients returned in list anti-HCV-neg, $p = 0.001$; patients on the waiting list anti-HCV-neg *vs* patients returned in list anti-HCV-neg, $p = 0.043$; control group anti-HCV-pos *vs* patients on the waiting list anti-HCV-pos, $p = 0.171$; control group anti-HCV-pos *vs* patients returned in list anti-HCV-pos, $p = 0.292$; patients on the waiting list anti-HCV-pos *vs* patients returned in list anti-HCV-pos, $p = 0.972$.

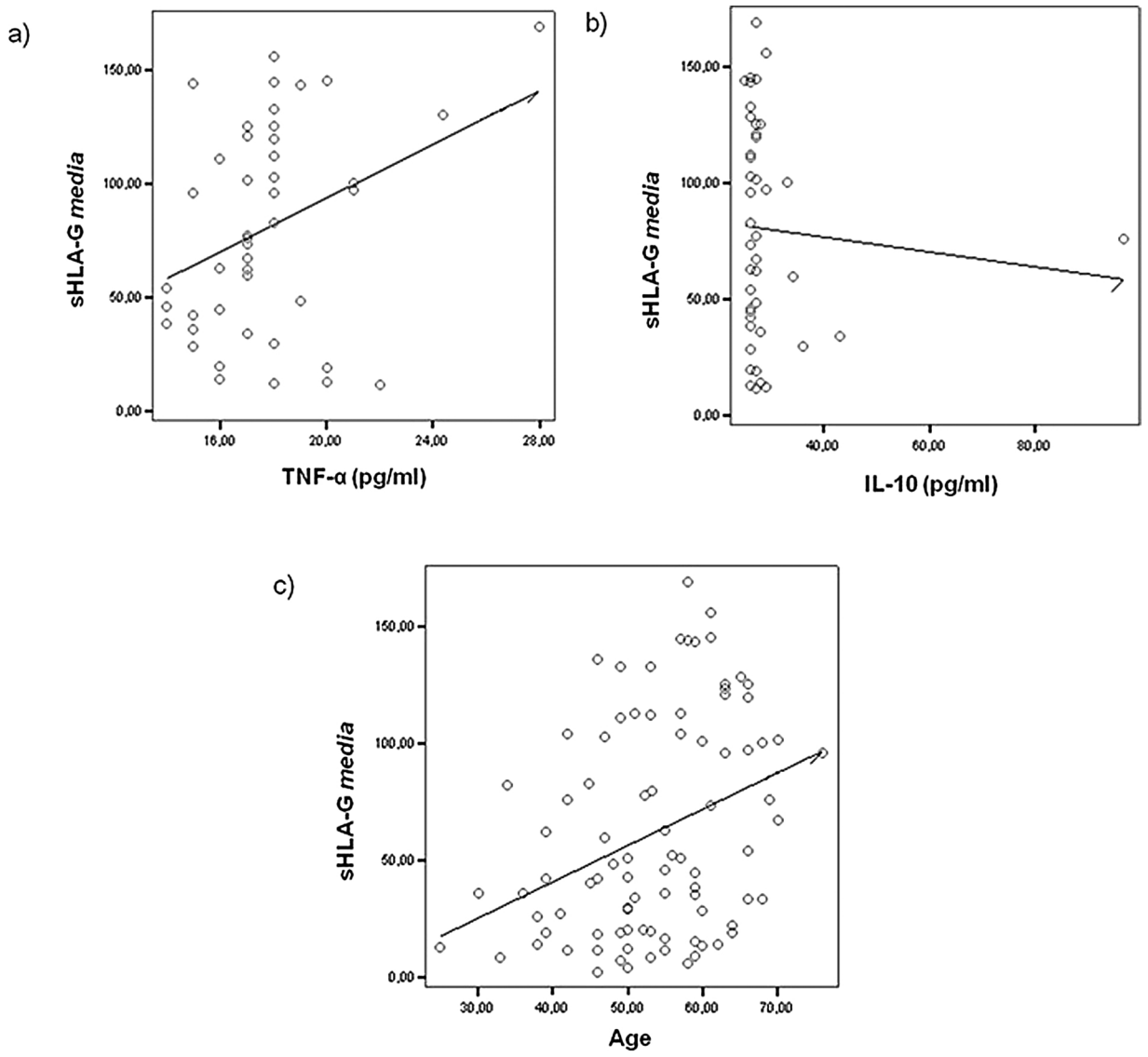

Fig. 3. Linear correlation between serum soluble human leukocytes antigen-G and TNF-α (a), IL-10 (b), and age (c).

the waiting list with both anti-HCV negative ($p = 0.001$) and between patients on the waiting list *vs* patients who returned on the list both anti-HCV negative ($p = 0.043$) (Fig. 2). No significant differences were observed in all anti-HCV positive groups.

In Fig. 3(a–c) we report the linear correlation of sHLA-G in relation to TNF-α, IL-10, and age. In particular, we observed a positive linear correlation between sHLA-G and TNF-α, and age (Fig. 3a and b). A negative correlation was found between HLA-G and IL-10 (Fig. 3c).

In Fig. 4 we report the linear correlation of HLA-G and TNF-α, age and IL-10 in the absence and presence of anti-HCV.

4. Discussion

Our study demonstrated that serum sHLA-G values were significantly increased both in patients on the waiting list for the first transplantation and in those who returned in the list after the first transplantation as compared to the control group (p for trend of sHLA-G = 0.004). Our data are completely in accordance with previous reports [29,30]. The small non-significant difference of sHLA-G levels observed between patients who returned in the list and patients on the waiting list for kidney transplantation would seem to be in contrast to literature data published by others where the increased levels of sHLA-G serum/plasma are associated with improved graft acceptance and its expression is considered as biomarker also in pre-transplantation [26–30]. Some clinical factors such as time elapsed since transplantation and antibody induction therapy are probably responsible for the discrepancies observed in our study [26]. Additionally, despite the high levels of sHLA-G were in relation to allograft acceptance it was reported that 11% of patients with recurrent rejection showed high levels of HLA-G expression in biopsy specimens [33]. A dynamic expression of sHLA-G antigen in the serum of patients with kidney transplantation was demonstrated [27]. Discordant data regarding the HLA-G expression could be due to the high variability of the methods for SHLA-G measurement. Indeed, HLA-G-specific ELISA does not discriminate between monomer or dimer isoforms [34]. Most studies on the expression of HLA-G by ELISA did not include any biochemical confirmation that detected antigens correspond to the HLA-G isoforms described [35].

Another cause of discordant data could probably be a correlation to a specific immune transcriptomic profile that discriminates chronic kidney disease patients. Indeed, an abnormal accumulation of pro-inflammatory cytokines would be observed in these patients with a consequent reduction of cytokines and increase of uremic toxins, elevated oxidative stress and increase of sepsis [36]. In addition, HLA-G gene expression is tightly regulated at both the transcriptional and post-

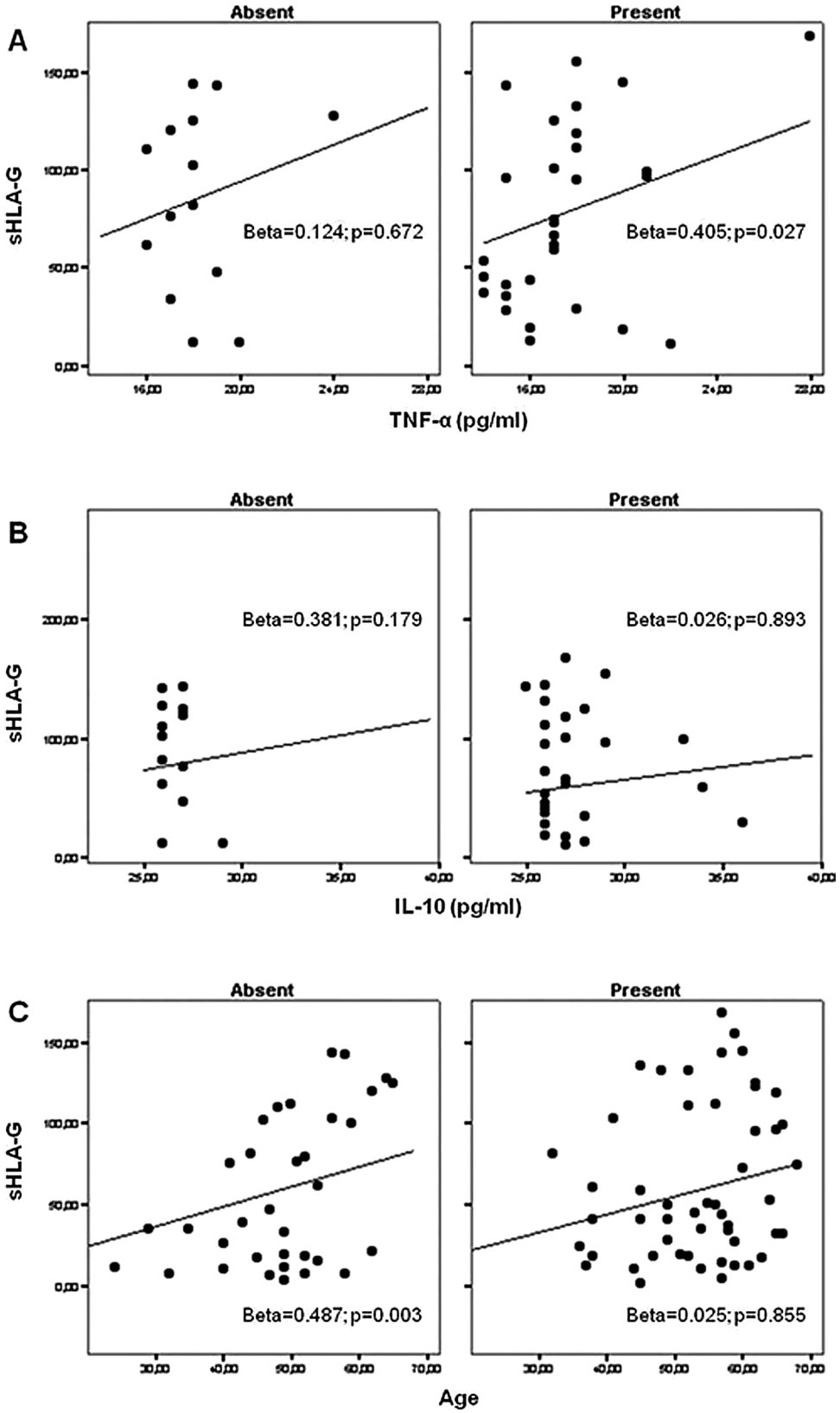

Fig. 4. a) Linear correlation of serum soluble human leukocytes antigen-G and TNF-α (a), IL-10 (b), and age (c) in the absence and presence of anti-HCV.

transcriptional levels and through epigenetic mechanisms such as DNA methylation and histone deacetylation [37]. The epigenetic mechanisms may play an important role in the regulation of HLA-G expression suggesting that it might be an important step in the activation of the HLA-G gene in some pathological situations such as inflammatory processes and cancer [2–6,38].

Regarding the correlation between sHLA-G and the presence of anti-HCV no significant difference was observed in all anti-HCV positive groups. All patients included in our study were NAT negative; thus, none of our patients showed active infection. Otherwise, we observed a positive linear correlation between sHLA-G and TNF-α in relation to presence of anti-HCV, and age in relation to absence of anti-HCV while no significant correlation with IL-10 was found in either group of patients. The role of cytokines played in the progression of HCV infection is still controversial. Some data suggest that IL-10 and HLA-G may regulate host immune response to HCV [17,18], no correlation was established in both for IL-10 and IFN-α and the expression of sHLA-G indicating that an increased sHLA-G production may not be induced by IL-10 and IFN-α in HCV infection [16].

5. Conclusions

There are important questions that should be answered in order to understand the biology of sHLA-G and clarify the results of the present study. Our study needs to be confirmed in a major population with a descriptive pre-clinical status and a careful long-term follow-up. Then, a cohort of both pre- and post-kidney transplant patients with acute rejections would be considered. In conclusion, our preliminary data highlight the need to improve the current HLA-G ELISA assays to obtain standardized methods useful in the clinical setting of transplant patients.

Conflicts of interest

The authors declare no conflicts of interests.

Financial disclosure

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Ethics information

Although our work was conducted under conformity of Declaration of Helsinki and no additional blood samples were taken, for all activities including the waiting list for organ transplantation and/or blood donation the approved Number of Ethic Committee, University of Campania “Luigi Vanvitelli” is Number: 295.

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