Longitudinal Tear Protein Changes Correlate with Ocular Chronic GVHD Development in Allogeneic Hematopoietic Stem Cell Transplant Patients

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Featured Application: This study reports for the first time an individual proteomic analysis of tears in patients pre-post HSCT, earlier than oGVHD onset. Total protein content, Lactoferrin, Transferrin, and Zinc-alpha-2-glycoprotein pre-post changes might be significant predictors of oGVHD development. Future efforts should further explore and support the appropriateness of incorporating tear proteome analysis in the clinical work up.

Abstract: Ocular graft-versus-host disease (oGVHD) is a manifestation of chronic GVHD, frequently occurring in patients after allogeneic hematopoietic stem cell transplant (HSCT). We analyzed tear protein changes before and after allogeneic HSCT, and correlated their levels with the oGVHD development. This retrospective study included 102 patients, and data were recorded before the conditioning treatment, and after 3 to 6 months postoperatively. Tear protein analysis was performed with the Agilent-2100 Bioanalyzer on individual tears sampled by aspiration. Total protein (TP), Lysozyme-C (LYS-C), Lactoferrin (LACTO), Lipocalin-1 (LIPOC-1), Transferrin (TRANSF), Albumin (ALB), and Zinc-alpha-2-glycoprotein (ZAG-2) levels were retrieved and statistically analyzed. Following HSCT forty-three patients developed oGVHD. TP, LACTO, LYS-C, and ZAG-2 levels significantly decreased post-HSCT as compared to pre HSCT levels. In univariate analysis, TP, LACTO, and ZAG-2 decrease was associated with an increased development of oGVHD (OR = 4.49; 95% CI, 1.9 to 10.5; $p < 0.001$; OR = 3.08; 95% CI 1.3 to 7.6; $p = 0.01$; OR = 11.1; 95% CI 2.7 to 46.6; $p < 0.001$, respectively). TRANSF post-HSCT levels significantly increased (OR 15.7; 95% CI, 4.1 to 52.2; $p = 0.0001$). No pre-post-HSCT changes were shown in ALB and LIPOC-1 levels. Data suggest that TP content, LACTO, TRANSF, and ZAG-2 pre-post changes might be significant predictors of oGVHD development.

Keywords: tear proteins; ocular GVHD; HSCT; odd ratio; lactoferrin; lysozyme; albumin; transferrin; lipocalin-1; zinc-alpha-2-glycoprotein; proteomic

1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a therapeutic choice for a large group of hematological, autoimmune, and hereditary disorders [1], but the onset of graft-versus-host disease (GVHD) may limit its outcomes, leading to mortality postoperatively [2]. GVHD may occur in 30 to 70% of patients postoperatively and is the result of a highly complex immune process, yielding to development of immune-mediated inflammation of target tissues and organs [3].

Ocular (o)GVHD is a frequent manifestation of chronic GVHD after HSCT [4,5], characterized by severe dry eye disease (DED) conditions associated with fibrosis of lacrimal and meibomian glands, superficial punctate keratopathy, corneal neovascularization, infectious keratitis, and even perforation [6]. The involvement of ocular surface post-HSCT has been reported, with a main focus onto the changes of tear parameters, including quantity, stability, and osmolarity, of surface epithelia, and of meibomian glands, in patients with or without oGVHD [7–13].

Tear biomarkers in oGVHD have focused mainly on the role of cytokines [8,10,14–17] and less on protein profiling to find candidate proteins that can be associated with the disease [18,19]. All these previous studies have been performed on post-HSCT patients, whereas ocular surface changes and high DED prevalence were shown already before HSCT [20,21]. This had suggested that a pre-HSCT ocular surface assessment is recommended for early DE management and appropriate evaluation of post-HSCT changes [6].

The purpose of this study was to analyze individual tear protein changes after allogeneic HSCT as compared to pre HSCT levels, and correlate their changes with the risk of later oGVHD development.

2. Materials and Methods

This single-center retrospective study included adult patients who received a first allogeneic HSCT between January 2010 and April 2020 at the Hematology Unit of the IRCCS AOU di Bologna, Policlinico di Sant'Orsola (Bologna, Italy) and underwent subsequent ocular surface examinations at the Ophthalmology Unit of the same hospital. The study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the local Ethics Committee (Comitato Etico di Area Vasta Emilia Centro della Regione Emilia-Romagna).

Exclusion criteria were: survival <100 days after transplantation, presence of other ocular surface disorders or any systemic disease potentially affecting the ocular surface at the time of HSCT, missing ophthalmological data after HSCT.

Data collected on charts and related to HSCT included the source of stem cells (bone marrow, peripheral blood or cord blood), type of donor (matched related—MRD or matched unrelated donors—MUD), conditioning regimen (myeloablative or reduced-intensity regimen based on patient age, previous treatments, comorbidities and status of malignancy). For the statistical analysis, patients were divided into two groups according to the underlying disease: chronic lymphoproliferative disorders (LPDs) including Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), multiple myeloma (MM), chronic lymphocytic leukemia (CLL) and stem cell malignancies (SCMs) including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndrome (MDS), and chronic myeloid leukemia (CML). All patients had undergone GVHD prophylaxis with either cyclosporine and methotrexate or cyclosporine and mycophenolate mofetil. Demographical and hematological data including age, sex, primary hematological disorder, age and sex of donor, presence of sex and HLA mismatch were recorded for each patient.

Grading of acute GVHD was performed in a 0 to IV scale according to the Glucksberg classification [22]. The severity of chronic GVHD was scored using the NIH 2014 criteria [23].

Data retrieved on ocular surface parameters included those collected before HSCT 7 to 9 days before the beginning of the conditioning regimen, and those collected in the first subsequent ophthalmological examination performed 100 to 180 days post-HSCT. Data included subjective ocular discomfort symptoms scored by using the ocular surface disease index (OSDI) validated questionnaire [24], tear film break-up time (TBUT), corneal staining, and Schirmer test. Tear film break-up time was evaluated after administration of 2 μL of 2% fluorescein dye and measuring the time interval between the last complete blink and the first appearance of a dry spot or disruption in the tear film. Corneal staining was graded using the National Eye Institute score (NEI Grading 2014). Schirmer test was performed without anesthesia using test strips kept into the temporal lower conjunctival sac for 5 min with closed eyes.

The diagnosis of ocular GVHD was based on the International Consensus Criteria on Chronic Ocular GVHD Group, which assign a 0–3 scoring point to Schirmer test, corneal fluorescein staining and OSDI, and 0–2 scoring point to conjunctival injection [25]. However, these criteria were introduced only in 2013, and we did not routinely score conjunctival injection before then. Thus, we employed modified criteria without conjunctival injection, and reduced by 1 point the aggregate score required for reaching the diagnosis: in the presence of systemic GVHD, score $\geq$ 5 indicated ocular GVHD (oGVHD); in the absence of systemic GVHD, score $\geq$ 7 indicated oGVHD [6]

Tear sampling procedure. A minimum amount of 10 µL of unstimulated tears was collected using a micropipette with sterile tips, avoiding any reflex tearing [26], from each subject in both eyes. Subjects were requested to position the head slightly reclined in such a way that tears are gathered at the most outer side of the lower fornix, avoiding tear drainage through the naso-lacrimal duct. After 30 s, the sterile disposable tip of a laboratory micropipette (Pipetman P, Gilson Int.l B.V., Den Haag, The Netherlands) was carefully positioned and tears were collected into a low protein absorption plastic vial, centrifuged at $13200\times g$ for 15 min, and the aspirated supernatant was subsequently stored in plastic vials at -80 °C until measurement. Sampling occurred in an interval ranging 8.30–9.30 a.m.

Tear analysis. Protein analysis was performed in individual samples with the 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA, USA, P230 Lab-chip kit) as described in detail elsewhere [26,27]. Total protein content (TP) and the following proteins were recognized and quantified: Total protein (TP), Lysozyme-C (LYS-C), Lactoferrin (LACTO), Lipocalin-1 (LIPOC-1), Transferrin (TRANSF), Albumin (ALB), and Zinc-alpha-2-glycoprotein (ZAG-2) levels. Proteins were expressed either as % versus total protein content and as µg/µL tear sample.

Statistical analysis was performed using IBM Statistical Package for social sciences version 26. For statistical analysis, normality was tested with Shapiro–Wilk test and non-parametric tests were considered. Spearman's (*p*) correlation coefficient was calculated between data from tear analysis and hematological or transplant parameters, correlations were considered statistically significant at $p < 0.05$ and a correction of *p*-values for multiple testing was introduced. Strength of correlation ranged from -1 to $+1$ and was estimated in absolute values as 0–0.19 "very weak"; 0.20–0.39 "weak"; 0.40–0.59 "moderate"; 0.60–0.79 "strong"; 0.80–1.0 "very strong".

The Wilcoxon matched-pairs signed rank test was performed to compare the different level of proteins before and after HSCT. Mann–Whitney U test was used to compare the difference in levels of proteins before and after HSCT (Δ pre-post) between patients with and without oGVHD.

Receiver operating characteristic (ROC) curves were drawn to assess the accuracy of tear proteins by using the MedCalc software (v19, MedCalc Software Ltd.). The accuracy of Δ pre-post for each tear protein, discriminating patients developing or not oGVHD, was evaluated by calculating the area under the curve (AUC), estimated as it follows: 0.9–1.0 excellent, 0.8–0.9 very good, 0.7–0.8 good, 0.6–0.7 sufficient. The optimal cut-off values of each tear protein were identified with Youden's index and tested with Chi square test. Risk for oGVHD was also estimated by odds ratio (OR) and risk ratio (RR) with 95% confidence intervals that independently predicted the disease.

p values less than 0.05 were considered statistically significant.

3. Results

A total of 102 patients (62 males and 40 females) were included in the study. Clinical hematological characteristics and demographic data are summarized in Table 1.

Table 1. Clinical and demographic data in subjects included in the study. Data are expressed as median (min-max values) (95% CI).

Demographic Data		Patient Number	% vs. Total
Females		40	39.2
Males		62	60.8
Age (yrs.) all		47.5 (18–65) [44.6–50.3]	
Females		49.0(21–61) [45–53]	
Males		47 (18–65) [41–50]	
Haematological History			
	Disorders		
SCMs	ALL	26	25.4
	AML	30	29.4
	MDS	10	9.8
	CML	6	5.8
LPDs	HL	10	9.8
	NHL	12	11.7
	MM	6	5.8
	CLL	2	1.9
Time from diagnosis to HSCT (days)		265 (123–2432) [183–421]	
Previous chemotherapy			
≤3 cycles		62	60.7
>3 cycles		40	39.3
HSCT Parameter			
Donor characteristics			
Age (yrs)		29 (0–56) [27–33]	
MUD		80	78.4
MRD		22	21.6
HLA mismatch		58	56.8
Sex mismatch		45	41.1
Conditioning regimen			
Reduced		24	23.5
Mieloablative		78	76.6
Stem Cell source			
Bone marrow		28	27.4
Peripheral Blood		68	66.6
Cord Blood		6	5.8

ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia, HL = Hodgkin lymphoma, CML = chronic myeloid leukemia, NHL = non-Hodgkin lymphoma, MM = multiple myeloma, MDS = myelodysplastic syndrome, CLL = chronic lymphocytic leukemia. MUD = matched unrelated donors; MRD = matched related donors; LPDs = chronic lymphoproliferative disorders; SCMs = stem cell malignancies.

Following HSCT, 20 patients (19.6%) developed acute GVHD, and 35 patients (34.3%) developed chronic GVHD. Forty-three patients (42.1%) developed oGVHD; the median time from HSCT to the onset of oGVHD was 332 days. Data were retrieved on a mean time of 151 ± 43 days post operatively. Ocular parameters were compared between No oGVHD and oGVHD and summarized in Table 2, but no statistically significant difference was found between groups.

Table 2. Summary of ocular parameters analyzed in patients with or without oGVHD post-hemopoietic stem cell transplant (HSCT). Data are expressed as median (min-max) [95% CI]. OSDI = Ocular Surface Disease Index; NEI = National Eye Institute.

Ocular Parameter	Not oGVHD	oGVHD	*p*
OSDI score	12.0 (0–58.0) [8.0–17.0]	17.0 (0–50.0) [9.3–20.6]	0.3
Schirmer test (mm length 5′)	20.0 (3.0–40.0) [19.1–30.0]	18.0 (2.0–40.0) [15.0–24.6]	0.3
TBUT (seconds)	7.5 (2.0–15.0) [6.1–8.2]	7.0 (1.0–15.00) [6.0–8.2]	0.4
NEI score corneal staining	1.0 (0–10.0) [1.0–2.0]	1.5 (0–12.0) [1.2–3.1]	0.4

Data from tear analysis performed pre-HSCT and post-HSCT are summarized in Table 3. TP, LACTO, LYS-C, and ZAG-2 levels significantly decreased post-HSCT as compared to pre HSCT levels ($p < 0.0001$). TRANSF post-HSCT levels significantly increased ($p < 0.0001$). ALB and LIPOC-1 levels did not exhibit statistically significant pre-post-HSCT changes.

Table 3. Levels of tear proteins analyzed in patients pre- and post-hemopoietic stem cell transplant (HSCT). Data are expressed as median (min-max) [95% CI].

Protein Name	Pre-HSCT (µg/µL)	Post-HSCT (µg/µL)	*p*
Total protein (TP)	7.4 (2.7–16.6) [6.7–8.1]	5.5 (1.3–11.2) [4.9–5.8]	<0.0001
Albumin (ALB)	0.9 (0.1–2.6) [0.7–1.1]	1.1 (0.1–3.1) [0.9–1.5]	0.5
Lysozyme-C (LYS-C)	2.2 (0.5–5.7) [2.0–2.4]	1.8 (0.3–4.5) [1.6–2.0]	<0.001
Lactoferrin (LACTO)	1.6 (0.1–4.0) [1.4–2.0]	1.2 (0.1–2.9) [1.1–1.4]	<0.0001
Lipocalin-1 (LIPOC-1)	1.6 (0.5–3.0) [1.5–1.7]	1.3 (0.4–2.5) [1.1–1.6]	0.05
Transferrin (TRANSF)	0.2 (0.1–1.4) [0.2–0.2]	0.5 (0.1–1.4) [0.4–0.5]	<0.0001
Zinc-alpha-2-glycoprotein (ZAG-2)	0.4 (0.1–1.1) [0.3–0.5]	0.2 (0.1–0.7) [0.1–0.2]	<0.0001

The comparison of levels in tear proteins before and after HSCT between patients developing or not oGVHD is summarized in Table 4. TP, LACTO, LYS-C, TRANSF, and ZAG-2, but not ALB and LIPOC-1, levels showed statistically significant differences in both groups.

The intra-subject individual pre-post difference (Δ pre-post) was calculated, and compared by stratifying patients into groups, developing or not oGVHD. Δ pre-post-ALB, LIPOC-1, and LYS-C were not found significantly different in the two groups ($p = 0.8, 0.9, 0.8$, respectively). Figure 1 summarizes the results from Δ pre-post TP, LACTO, TRANSF, and ZAG-2, that were found statistically significantly higher in the group developing as compared to the group not developing oGVHD.

Table 4. Comparison of levels of tear proteins analyzed pre and post-hemopoietic stem cell transplant (HSCT) in patients with or without ocular graft versus host disease (oGVHD). Data are expressed as median (min-max) [95% CI]. TP = total protein; ALB = Albumin; LYS-C = Lysozyme-C; LIPOC-1 = Lipocalin-1; TRANSF = Transferrin; ZAG-2 = Zinc-alpha-2-glycoprotein.

| Tear Protein	No oGVHD			oGVHD		
	Pre-HSCT (µg/µL)	Post-HSCT (µg/µL)	*p*	Pre-HSCT (µg/µL)	Post-HSCT (µg/µL)	*p*
TP	7.2 (2.7–14.5) [6.7–8.1]	5.8 (2.5–11.1) [4.9–5.8]	<0.001	7.4 (3.2–16.2) [6.8–8.6]	4.8 (1.3–11.2) [3.8–5.7]	<0.0001
ALB	0.9 (0.1–2.6) [0.7–1.1]	1.3 (0.1–3.1) [0.8–1.5]	0.5	0.9 (0.1–2.5) [0.7–1.2]	1.1 (0.1–2.6) [0.7–1.4]	0.5
LYS-C	2.2 (0.4–4.5) [2.0–2.5]	1.8 (0.7–4.5) [1.6–2.1]	<0.001	2.0 (1.2–5.7) [1.8–2.4]	1.8 (0.3–4.4) [1.4–2.0]	<0.1
LACTO	1.1 (0.1–3.7) [1.2–1.8]	1.2 (0.4–2.8) [1.1–1.4]	<0.01	1.9 (0.4–4.0) [1.4–2.3]	1.2 (0.1–2.9) [1.0–1.5]	<0.0001
LIPOC-1	1.5 (0.5–2.3) [1.2–1.7]	1.3 (0.3–2.5) [1.9–1.6]	0.07	1.6 (0.6–3.0) [1.5–1.8]	1.5 (0.5–2.1) [1.1–1.7]	0.1
TRANSF	0.2 (0.1–0.5) [0.1–0.2]	0.4 (0.1–0.8) [0.3–0.5]	<0.0001	0.2 (0.1–1.4) [0.2–0.2]	0.7 (0.1–1.4) [0.5–0.7]	<0.001
ZAG-2	0.4 (0.1–0.7) [0.3–0.4]	0.2 (0.1–0.7) [0.1–0.2]	<0.001	0.5 (0.1–1.1) [0.5–0.6]	0.2 (0.1–0.5) [0.1–0.3]	<0.001
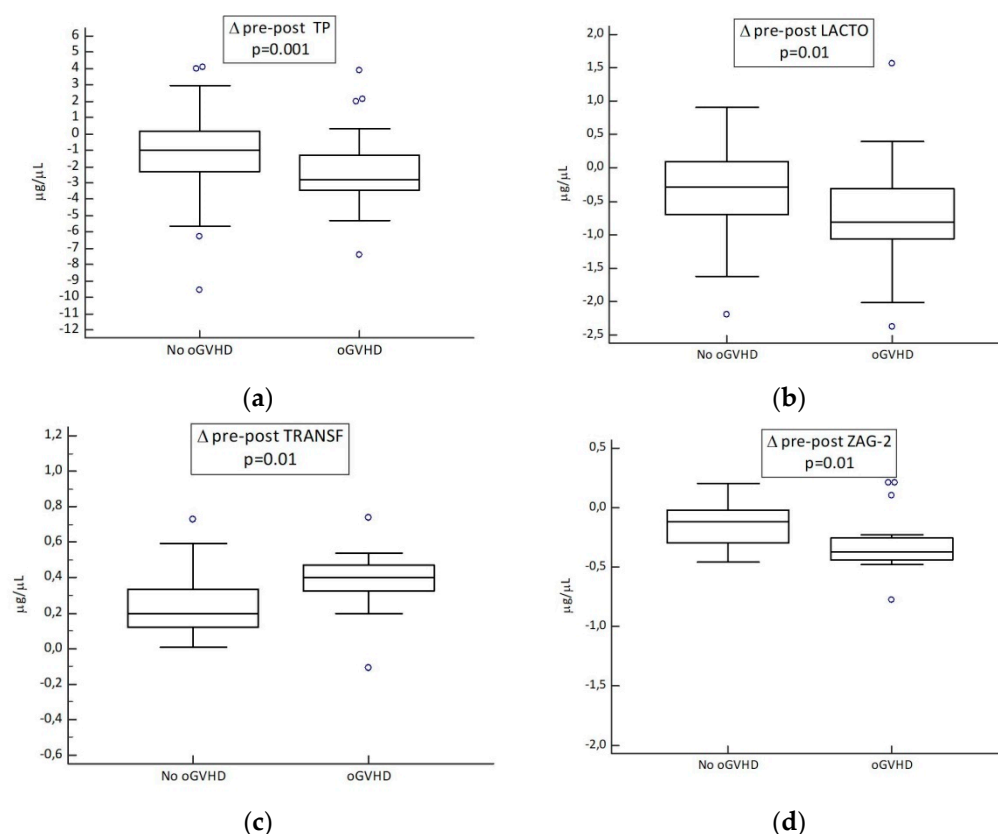
Figure 1. Boxplot representation of pre-post changes in the levels of TP (**a**), LACTO (**b**), TRANSF (**c**), and ZAG-2 (**d**), expressed in µg/µL. Statistically significant differences are present between groups of patients developing ocular graft versus host disease (oGVHD) or not (No oGVHD), for all proteins.

Figure 2 summarizes the receiver operating characteristic (ROC) curve analyses performed for Δ pre-post-TP, LACTO, TRANSF, ZAG-2. The area under the curve (AUC)

values showed the cut-off of −1.86 μg/μL for Δ pre-post-TP and −0.45 μg/μL for Δ pre-post-LACTO sufficient to discriminate between patients developing or not oGVHD. The cut-off of 0.34 μg/μL for Δ pre-post-TRANSF and of −0.23 μg/μL for Δ pre-post-ZAG-2 was good in discriminating between these two groups.
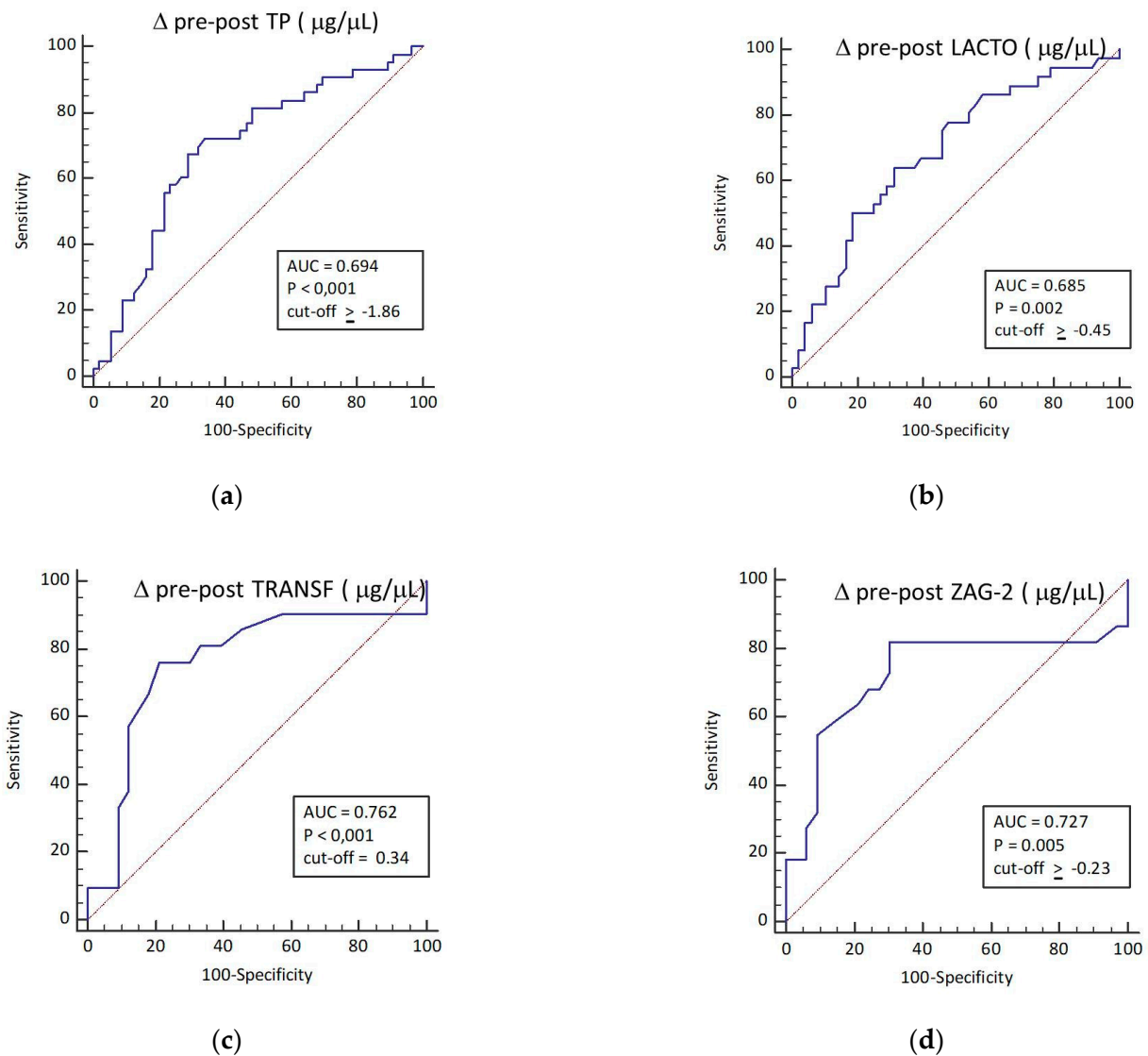

Figure 2. ROC curve analysis performed for Δ pre-post-TP (**a**), LACTO (**b**), TRANSF (**c**), and ZAG-2 (**d**). The area under the curve (AUC) values and the cut-off values are listed in all panels, along with the *p* values, always statistically significant.

A statistically significant correlation was not found between tear protein levels and hematological or transplant parameters. No correlation was found with the presence of aGVHD. Sex mismatch and mieloablative conditioning regimen appeared to correlate with higher Δ pre-post-TP, but with a significance of *p* = 0.05.

In univariate analysis an association was found between oGVHD onset and Δ pre-post-TP, LACTO, TRANSF, and ZAG-2. Table 5 summarizes odd ratio and risk ratio for Δ pre-post-TP, LACTO, TRANSF, and ZAG-2, which were identified as significant risk factors for oGVHD (respectively, OR 4.49, 3.08, 15.7, and 11.1).

Table 5. Odd ratio and risk ratio for oGVHD onset of the difference pre-post-HSCT of tear total protein (TP), lactoferrin (LACTO), transferrin (TRANSF), and zinc-alpha-2-glycoprotein (ZAG-2).

Factor	Cut-Off (µg/µL)	OR 95% CI	p	RR 95% CI	p
Δ pre-post-TP	≥−1.86	4.49 [1.9–10.5]	<0.001	2.1 [1.3–3.1]	<0.001
Δ pre-post-LACTO	≥−0.45	3.08 [1.3–7.6]	<0.01	1.8 [1.1–3.1]	0.01
Δ pre-post-TRANSF	>0.34	15.7 [4.1–52.2]	0.0001	3.8 [1.9–7.6]	0.0001
Δ pre-post-ZAG-2	≥−0.23	11.1 [2.7–45.6]	<0.001	2.3 [1.5–3.4]	<0.001

4. Discussion

Data from the present study show that individual pre-post-HSCT changes in tear protein levels, in particular TP, LACTO, TRANSF, and ZAG-2, estimated in the first six months postoperatively are correlated to the late onset of ocular GVHD. This finding may be of clinical impact for the early management of serious ocular complications following HSCT.

To the best of our knowledge, the present paper is the first one dealing with individual changes of tear protein levels from baseline conditions before the conditioning protocol to conditions recorded in the interval 100–180 days after, thus introducing consistency in the time frame of comparison.

Ocular GVHD represents a current diagnostic and therapeutic challenge, with increasing prevalence and severe involvement of the ocular surface with vision-threatening complications, and a dramatic reduction of the quality of life [28]. Therefore, an early diagnosis driving an appropriate and prompt treatment is important for avoiding the occurrence of severe complications [29].

Current diagnostic criteria of oGVHD include clinical parameters (such as the Schirmer test, the corneal damage evaluated by vital staining, conjunctival hyperemia, and the subjective discomfort symptoms) participating with different scoring points to a global score assessment [2,25,30,31]. However, the well-known lack of causal correlation between clinical findings and subjective symptoms may hinder and delay the oGVHD recognition, and the search for biomarkers as objective diagnostic and monitoring tools is an urgent need. Data from the present study did not evidence statistically differences in ocular surface clinical parameters and subjective symptom scores in patients developing oGVHD as compared to those not developing oGVHD.

Tears are good candidates as a body fluid to identify biomarkers for the management of patients suffering from not only ophthalmological diseases [32–37], as they are close to the disease site, offer analytical advantages, and their sampling technique is relatively not invasive.

Due to the extensive inflammatory processes underlying the GVHD onset and progression, several works have been addressed onto tear cytokine content, with the aim to differentiate patients with oGVHD from healthy or DED subjects [8,10,14,15] or from patients without oGVHD [17], or without any control [16]. Apart from technical issues deriving from a large variability in pre-analytical and analytical procedures [38], all these investigations have been performed in patients only after HSCT and at different periods postoperatively. Similar attempts had been made on plasma cytokine panels for their potential to predict the development of GVHD [39,40].

Tear proteomic-based studies were also performed to discriminate down or up regulated proteins, distinctive for oGVHD as compared to non oGVHD [18,19], but again only in post-HSCT patients. Indeed, several proteomic approaches are currently used in the prediction or diagnosis of acute and/or chronic GVHD [41–43]. Interesting preliminary results are now available from the research setting, but without validation and impact on clinical decision making so far.

The present paper introduces a relatively unexplored field for biomarker search in oGVHD, dealing with proteins mainly secreted by the disease target tissues of the ocular surface, and collected in tears.

Currently, almost 1800 proteins are known to constitute the human tear proteome, and some panels for up or down-regulation in DED have been recently proposed [44]. To briefly review tear proteins analyzed in this paper, tear LACTO along with LYS-C and LIPOC-1 are co-localized with in the lachrymal gland secretory granules, they might most likely be secreted together, suggesting a role as indicators for the lachrymal gland function [45]. In particular, LACTO and LYS-C are multifunction chain polypeptides with anti-inflammatory, bacteriostatic, and antioxidant properties, whereas LIPOC-1 is the main lipid carrier and scavenger in human tears and is crucial in the ocular surface protection [45–47]. A recent meta-analysis suggested LACTO level in tears is a good candidate as dry eye syndrome diagnostic biomarker [48]. The plasma-derived proteins ALB and TRANSF can be considered as indirect sign of subclinical inflammation as it occurs when there is an increase of protein leakage from inflamed conjunctival vessels [49–51]. They are usually present in a negligible amount in tears of normal subjects [26], and it has been shown that ALB increases whereas TRANSF decreases in moderate DED [27]; it is not clear why these proteins behave contrarily and if this may be due to blood-epithelial barrier functioning. First reported in human serum and later in other body fluids, ZAG-2 represents an interesting protein that stimulates lipid breakdown in adipocytes, and which is highly expressed in cancer cachexia [52]. A reduction of ZAG-2 tear levels was documented in CL wearers [53], DED [27,54], fungal infections at an early stage [55–57], diabetic patients [58], and an increase in tears of smokers and patients with Grave's ophthalmopathy [59]. The function of ZAG-2 in tears and its possible role in lipid degradation has been postulated but not demonstrated [59].

A decrease of TP levels in the tears may generally indicate a reduction in synthesis capability by the lachrymal glands, as it has been hypothesized in Sjogren's patients [32]. Lacrimal gland dysfunction and fibrosis is a major feature of oGVHD [60], and this may explain the decreased level of the tear TP in our patients, with distinctive profiles identified in this longitudinal study earlier than the clinical diagnosis. Some results were in agreement with what was found previously in post-HSCT patients who had developed oGVHD [18,19], in particular the reduction of LACTO and LYS-C, whereas another major tear proteins such as LIPOC-1 appeared unmodified in our study. We hypothesize this might be related to the tear analysis performed early after HSCT, with oGVHD yet to come, and with a lachrymal gland still partially functioning and secreting LIPOC-1. Increased levels of plasma-derived TRANSF but not ALB might indicate an early modification of blood-epithelial barrier, and as above reported a sign of exudation and inflammation at a subclinical level.

Lowering of tear ZAG-2 in this study is unexpected, as these are cancer-suffering patients, where an increase of ZAG-2 plasma levels had been observed. The pre-post change of this protein seems to be highly correlated with the oGVHD development, so the findings from this work are a preliminary step to deepen ZAG-2 role in this progression. Taken as a whole, TRANSF and ZAG-2 changes are indicative of inflammation and lipid metabolism alteration, earlier than oGVHD onset. Parameters of ROC curves, in particular the AUC, for TRANSF and ZAG-2 appear to be less significant than those reported in [18], but in this paper the diagnostic accuracy for oGVHD had been analyzed versus healthy controls.

In conclusion, this study reports for the first time an individual pre-post proteomic analysis of tears from a relatively high number of patients, all transplanted and followed in the same referral center, making consistency in procedures and time frame. We have demonstrated distinctive profiles of pre-post changes of four analytes in tears, correlated to the later onset of oGVHD, and with considerable and significant odd ratio.

The clinical information for this study is that an increased risk for severe diseases of the ocular surface is present in post-HSCT patients earlier than oGVHD onset, and an appropriate ophthalmological work up should be established soon after the surgery. Future efforts should further explore and support the appropriateness of incorporating tear proteome analysis pre and post-HSCT.

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