

Evaluation of Potential Pain Biomarkers in Saliva and Pain Perception After Corneal Advanced Surface Ablation Surgery

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Purpose: To evaluate the evolution of a set of proposed pain biomarkers in the saliva of subjects following Advanced Surface Ablation (ASA), in order to determine their validity as objective pain measures.

Methods: A multicenter, prospective, and descriptive study was carried out to assess the variations between biomarkers and perceived pain. The Inclusion criteria were healthy subjects who underwent a bilateral, alcohol-assisted surface ablation with epithelial removal (ASA). Pain intensity before and after surgery was assessed by Visual Analog Scale (VAS) and the Numeric Pain Rating Scale (NPRS). Cortisol, sAA, sIgA, testosterone, and sTNF α R2 were assayed at four-time points (V0, baseline; V1, pre-surgery; V2, 1 hr post-surgery, and V3, 72 hrs post-surgery). Comorbidities and Hospital Anxiety and Depression (HADS) questionnaires were administered before and at 6 hrs after the surgery. All patients were treated with cold patches, topical steroids, topical cold antibiotics, and benzodiazepines after ASA surgery. A descriptive analysis of biomarkers and pain intensity evolution and the agreement between biomarkers and pain was performed.

Results: Concentration of sIgA and sTNF α R2 post-surgery was significantly higher at each visit compared to baseline (p -value: 0.053, p -value: <0.001, respectively). Relations between VAS scale score and putative biomarker variations were not statistically significant except for the sIgA but only at visit 0 (p -value: 0.024). The HADS questionnaire showed anxiety scores between 0 and 7 in all patients before and at 6 hrs after surgery.

Conclusion: In this study, sIgA and sTNF α R2 are the two potential biomarkers that present correlation with the VAS and these salivary substances showed acceptable levels of reproducibility in healthy subjects.

Keywords: biomarkers, ocular pain, advanced surface ablation

Introduction

Research into human pain has undergone strong development over the last 25 years and new biomarkers have emerged from different sources.¹ The identification of pain biomarkers is the third of the potential tools.² Saliva is a good biomarker for clinical applications. It is safe, easy, and non-invasive to collect and economic.³ The use of saliva as a reliable substitute for blood could provide an avenue for biomarker measurement in pain studies.^{4,5} During recent decades, saliva as a diagnostic and prognostic fluid in pain research has received increasing attention.⁶ It is common to find studies analyzing optimal saliva collection techniques for studying pain biomarkers, the most sensitive method for detecting and analyzing these biomarkers and,

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increasingly, more substances found in saliva, also present in blood and related to pain, are analyzed.^{6,7}

To date, the main potential pain biomarkers already described in saliva are soluble tumor necrosis factor- α receptor II (sTNF α RII),⁴ secretory immunoglobulin A (sIgA),⁸ cortisol, salivary alpha-amylase (sAA),⁹ and testosterone.¹⁰

Acute dental pain has been associated with an increase in salivary cortisol,⁷ acute abdominal disease in horses has produced an increase in sAA activity,¹¹ da Silva et al found a negative correlation between oral pain intensity and sIgA levels in children,⁸ Choi et al reported the effects of testosterone on pain,^{10,12} and Goodin et al has produced several studies analyzing sTNF α RII response to experimental modalities of acute pain.^{4,13,14}

A previous study conducted by our group analyzed these biomarkers, all together, in healthy subjects. The findings suggested that salivary sIgA and sTNF α RII show remarkable reproducibility.¹⁵ Owing to the controversy that exists regarding these substances as biomarkers of pain, and despite our previous findings, we decided to analyze all of them in patients with acute eye pain following Advanced Surface Ablation (ASA) surgery.

ASA is the term that grouped the techniques that have been continuously improved from the earlier photorefractive keratectomy (PRK). Today's ASA covers numerous techniques, such as laser epithelial keratomileusis (LASEK), epi-LASIK, and epi-LASEK.^{16,17} ASA procedures are safe and effective corneal refractive surgery techniques used to correct refractive errors.¹⁸ But one of their most important drawbacks is the discomfort and the presence of pain in the acute postoperative period.¹⁷ Postoperative acute ocular pain following ASA has been well characterized, with abundant information on the duration, intensity, and peak of pain following ASA in clinical practice.¹⁹ Therefore, the aim of the current study was to evaluate variations in the five potential pain biomarkers mentioned, as detected in the saliva of patients before and after ASA, to help determine their validity in measuring pain variations.

Materials and Methods

Ethics Statement

The study was approved by the local Ethics Committee of the Clinic University Hospital (Valladolid Spain) (PI 14-185, January 2015) and followed the tenets of the Declaration of Helsinki. Also, it complies with the Regulation 2016/679 of the European Parliament and of the Council of 27 April 2016 on the protection of natural persons with regard to the

processing of personal data and on the free movement of such data, as well as with the rest of the laws and regulations in force and applicable as Spanish biomedical research regulatory requirements. All patients received written informed consent before entering in the study. All subjects provided their written informed consent before participation in the study.

Design and Study Population

We conducted a multicenter, prospective, and descriptive cohort study.

The population for this study (32 consecutive patients from 2 different centers) is the same as that used in a previous study conducted by our group.¹⁹ Also, a detailed explanation of the exclusion and inclusion criteria has been provided in our previous publication.¹⁹ In brief, all participants were healthy subjects affected by myopia (0.75 to 9 diopters (D)) or hyperopia (0.25 to 5D) with or without astigmatism, who underwent bilateral ASA surgery. The exclusion criteria were: age under 18 years old, any pain-body in the preceding 7 days; previous ocular surgeries within the last 12 months; autoimmune diseases; previous treatment with painkillers (including anti-inflammatories), psychotropics, anticonvulsants, or antidepressants. Two experienced surgeons performed all the ASA surgeries.

Data on gender, ocular comorbidities, pain intensity, and rescue medication administered within 72 hrs after ASA surgery was recorded.

Hospital Anxiety and Depression (HADS) and quality of life (QoL) questionnaires were gathered before and 6 hrs afterward the surgery. Additionally, any post-operatively adverse events up to 7 days following the ASA surgery were registered.

ASA Technique

Detailed explanation of ASA procedure has been provided in our previous publication.¹⁹

In the preoperative period, all patients took 0.5 mg alprazolam 30 mins before surgery and one drop of 5% lidocaine was applied at least three times before ASA surgery (20 mins, 10 mins and just before the surgery).

In the intraoperative period, an ethyl alcohol solution (17%) was placed on the cornea within an 8.5-mm solution cone and left in place for 30 s. Then, a Merocel[®] sponge was used to absorb any excess fluid. Subsequently, topical cold balanced salt solution (BSS) was used to rinse the corneal surface, and the corneal epithelium was removed with a blunt spatula. This maneuver was followed by stromal ablation

using the MEL 70 G excimer laser (Carl Zeiss Meditec, Jena, Germany), which incorporates an eye-tracking system. The diameter of the treated area was always at least 6 mm with a transition to 9 mm.

After ablation, the surface was rinsed again with cold BSS. Then, 0.3% ofloxacin and 0.18% sodium hyaluronate drops were applied. At the end of the procedure, a therapeutic contact lens kept at 4°C was placed on the cornea.

In postoperative period, all patients stayed in the surgical facilities for 4 or 6 hrs following ASA. Promptly after surgery, patients received a cold patch over the lids for 15 mins. Afterward the surgery, subjects were given to apply cold topical antibiotics, cold topical steroids, topical 0.18% sodium hyaluronate, and artificial tears to both eyes during specific periods of time. Also, they were instructed to keep all eye drops at 4°C. Additionally, they were treated with oral alprazolam 0.5 mg and oral vitamin C tablets.

No other painkillers were prescribed unless the pain was deemed unbearable. In such cases, an established “rescue medication” protocol was used. Our rescue medication protocol was described in our previous publication.¹⁶

Postoperative Pain Assessment

Pain intensity was evaluated by using the Visual Analogue Scale (VAS) and the Numeric Pain Rating Scale (NPRS).²⁰ Pain intensity was evaluated prior to and 0.5, 1, 2, 3, 4, 5, 6, 9, 12, 24, 36, 48 (2nd day), 60, 72 (3rd day), 96 (4th day), 120 (5th day), and 144 (6th day) hrs after ASA surgery. Each patient was also instructed to record in an individual diary the medication usage, pain, or depression observations at home. This methodology was described in the previous study conducted by our group.¹⁹

Sample Collection and Analysis

The protocol on sample collection and analysis has been previously described in previous publications.^{15,21} In brief, 32 subjects were instructed on how to carry out the saliva collection using the passive secretion method²¹ over a 5-min period into a collection tube. The minimum amount of sample collected was at least 1 mL. If the 5-mL collection tube was filled before 5 mins, the amount of elapsed time was recorded. The samples with visible blood contamination were discarded and after a 10-min wait, new samples were collected.

The collection of samples was obtained from each subject, in a clinical setting and was always supervised by the investigators, in four visits: V0, baseline; V1, pre-surgery; V2, 1 hr post-surgery, and V3, 72 hrs post-surgery.

The following salivary biomarkers were assayed by enzyme-linked immunosorbent assay (ELISA) and analyzed using the commercially available kits: Cortisol (DRG[®] Salivary Cortisol ELISA, DRG[®] Instruments GmbH, Marburg, Germany), testosterone (DRG[®] Salivary Testosterone ELISA, DRG Instruments GmbH, Marburg, Germany), sAA (DRG Salivary Alpha Amylase ELISA, DRG Instruments GmbH, Marburg, Germany), sTNF α RII (Quantikine[®], Human sTNF RII/TNFRSF1B Immunoassay, R&D Systems, Minneapolis, MN, USA) and sIgA (Salimetrics[®] Salivary Secretory IgA ELISA, Pennsylvania, USA).

Other Ocular Symptoms, Hospital Anxiety/Depression (HADS) Questionnaire, Quality of Life (QoL) Questionnaire, and Visual Function 25 (VF25) Questionnaire

The text reproduces information already reported in detail in a study by Sobas et al.¹⁹

Other Ocular Symptoms

Basing on our previous developed questionnaire,²⁰ we have registered data on the presence and intensity of pain. Accompanying ocular symptoms such as burning sensation, photophobia, foreign body, tearing, itching, and headache were also recorded in the questionnaire. We rated the intensity of each item basing on the Likert scale of 4 points (range 0–3, where 0 corresponded to no symptom and 3 to the worst imaginable symptom).²²

These ocular symptoms were evaluated prior to, and at 1, 6, 48, and 120 hrs after surgery. All this information was included in the patient’s diary.

Hospital Anxiety/Depression (HADS) Questionnaire

The HADS scale, validated, and translated into Spanish language,²³ was used to assess pre- and post-surgical anxiety and depression. HADS was evaluated prior to, and at 6, 48, and 120 hrs following ASA.

Full details of HADS questionnaire are given in the following publication²⁴ but, briefly, patients completed a self-administered questionnaire composed of 14 items, divided into two 7-item subscales, one for anxiety and one for depression. Both subscales had the same cut-offs: 0–7, normal; 8–10, doubtful; and ≥ 11 , existence of a clinical problem.²⁵

Quality of Life (QoL) Assessment

QoL questionnaires were filled out by patients themselves. QoL was evaluated using the QoL short form-12 (SF12) health survey prior to, and at 24 and 72 hrs after surgery. This self-administered questionnaire consisted of 12 questions to measure physical and mental health status; a physical component summary (SF12 PCS) and a mental component summary (SF12 MCS). The score was computed from the scores of the twelve questions and ranged from 0 to 100. A score of 0 indicated the lowest level of physical and mental health, and a score of 100 indicated the highest level.²⁶

Visual Function 25 (VF25) Questionnaire

The VF25 questionnaire was administered prior to, and at 24 and 72 hrs after surgery. This questionnaire contains 25 questions within 11 vision subscales plus an additional single-item general health rating question. Scoring ranges from 0 (the lowest visual function) to 100 (best vision-related function).²⁶

Statistical Analysis

Sample size was based on our previously published postoperative pain study.¹⁹ We assumed that 98% of the participants would have ocular pain. A sample size of 32 subjects was determined for estimating the proportion of pain reporters, based on a two-sided 95% confidence interval (95% CI). The margin of error was set at $\pm 2.5\%$.

Statistical analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria). The significance level was set at 0.05.

Continuous variables were described as mean \pm standard deviation (SD) and categorical data were summarized as absolute frequency and percentages. Biomarker concentrations were analyzed as log2-transformed variables. sAA levels were below the detection limit in two visits to a subject. These values were imputed using the robust regression on order statistics (ROS) method implemented in the NADA R package.²⁷

Linear mixed-effects models using the R package lme4 were used to analyze the biomarkers' evolution.²⁸

Relationships between biomarker levels, gender, and age were evaluated at each visit. Student's *t*-test for two independent samples and Spearman's rank correlation were used, respectively. To evaluate the relationship with the VAS scale, two groups were

considered: 0 and >0 . Analysis of Variance (ANOVA) was performed to evaluate the differences between these groups in levels of each biomarker, adjusted by sex and age.

Results

Patient Characteristics

A total of 32 consecutive patients (19 men and 13 women) were included between June 2015 and June 2016. The mean age was 28.78 ± 6.93 and all patients received surgery on both eyes.

Pain Evolution After ASA Procedure

All patients reported postoperative ocular pain ($VAS \geq 20$ mm) from 0.5 to 60 hrs after surgery. The average pain intensity for all participants increased sharply during the first 9 hrs after surgery, and then peaked at 24 hrs (mean (SD) VAS scores of 61 (31) mm), with a 6-point difference between pre- and post-surgical VAS at that time (*p*-value: 0.012). It should be highlighted that the value at the mean (SD) time of 1 hr post-surgery was 4.91 (1.89) mm because it was at that moment when the biomarkers were analyzed. After that, the pain intensity was approximately stable until 36 hrs, then it slowly decreased up to 96 hrs after surgery. The mean (SD) VAS score at 72 hrs was 19 (20) mm.¹⁹

HADS Questionnaire

Before surgery, 29 (91%) of the patients scored between 0 and 7, [mean (SD) 4.62 (2.08)], 1 (3%) scored 10, and 1 (3%) scored ≥ 11 in the anxiety subscale. At 6 hrs after surgery, all patients had anxiety scores between 0 and 7 [mean (SD) 4.38 (2.5)]. For the depression subscale, all patients scored between 0 and 8 before surgery (1.16 ± 2.07). Six hours after ASA, the results were similar [mean (SD) 1.53 (2.09)].¹⁹

Values for All Biomarkers in the Saliva of Healthy Subjects

The basal salivary concentrations of cortisol, sAA, sIgA, testosterone, and sTNF α RII were previously assessed by our group in a prospective study involving 34 pain-free healthy subjects in two single samples at least 24 hrs apart.¹⁵ This research showed differences in potential biomarker concentrations between collections.

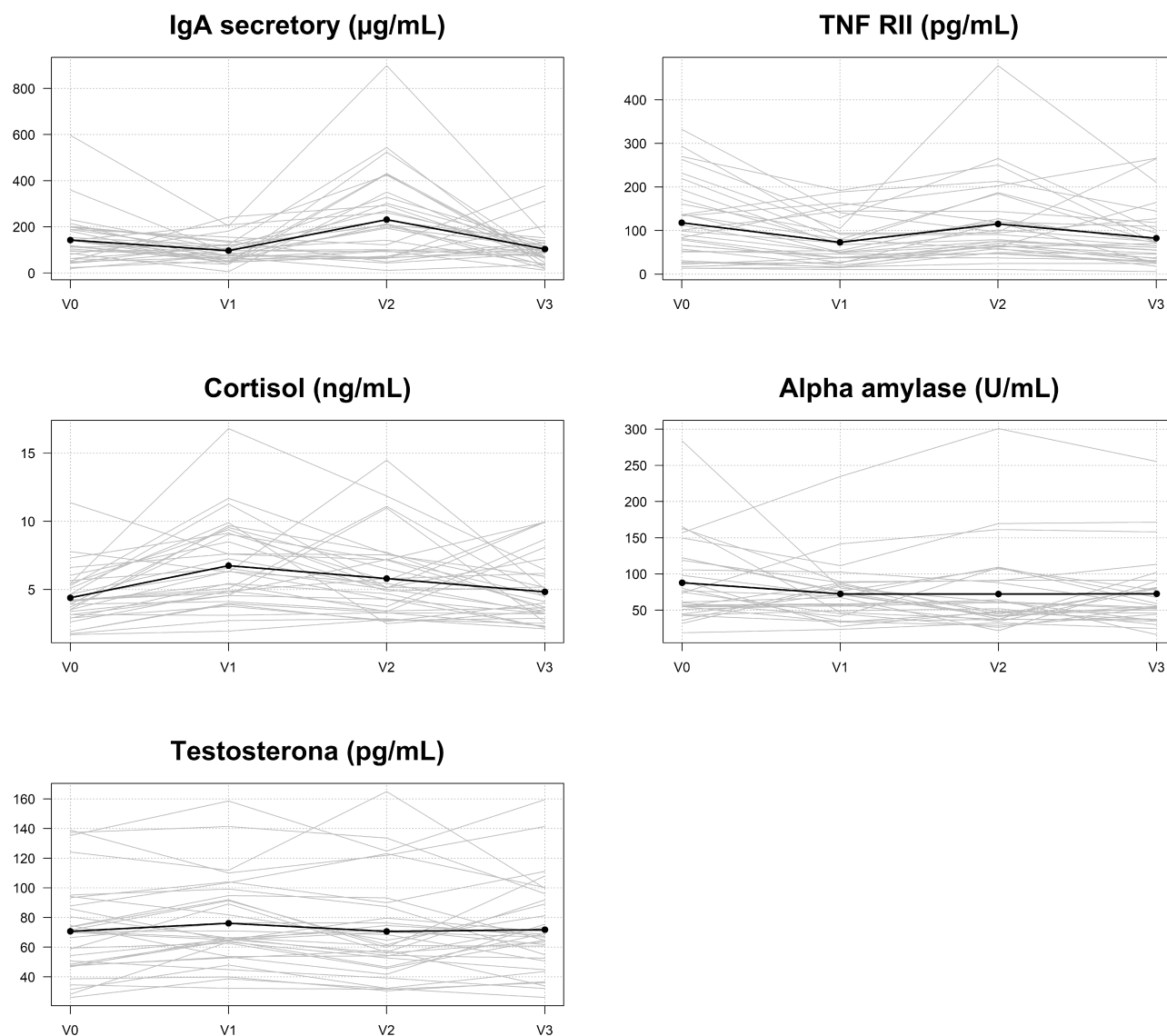


Figure 1 Biomarker concentration at four visits: V0, baseline; V1, pre-surgery; V2, 1 hr post-surgery, and V3, 72 hrs post-surgery. sIgA ($\mu\text{g/mL}$), sTNF α RII (pg/mL), cortisol (ng/mL), AAs (U/mL), testosterone (pg/mL).

Relationship of Biomarkers with Visits

Figure 1 shows biomarker concentration at four visits: V0, baseline; V1, pre-surgery; V2, 1 hr post-surgery, and V3, 72 hrs post-surgery.

The levels of sIgA, sTNF α RII, and cortisol were different at least two visits; the effect of the visit was significant. But sAA and testosterone remained at the limit of significance (Table 1).

Table 1 Effect of the Visit Extracted from the ANOVA Table for the Fitted Linear Mixed Models of Each Biomarker

	Sum of Squares	Mean Squares	gl Num	gl Denom	F-value	p-value
IgA visit	22.255	7.4182	3	93	6.6935	0.0003856
sTNF α RII visit	11.322	3.774	3	93	9.3961	1.741e-05
Cortisol visit	7.0011	2.3337	3	93	11.554	1.663e-06
sAA visit	2.2329	0.7443	3	93	2.2448	0.08824
Testosterone visit	0.47536	0.15845	3	93	2.3945	0.0733

Note: The bold text represents sIgA, sTNF α RII, and cortisol, were different at least two visits, the effect of the visit was significant.

Abbreviations: sIgA, secretory IgA; sTNF α RII, soluble fraction of receptor II of tumor necrosis factor α ; sAA, α -amylase.

Table 2 (A) Average Levels of sIgA at All Visits; **(B)** Difference Between Averages of Significant Effects in sIgA Levels at All Visits

(A)							
Effect		Log2			µg/mL		
		Est.	CI95% Est.		Est.	CI95% for Est.	
			Inf.	Sup.		Inf.	Sup.
Visit	V0	6.8	6.409	7.199	111.71	84.952	146.887
	V1	6.33	5.94	6.73	80.72	61.389	106.145
	V2	7.35	6.955	7.745	163.14	124.07	214.524
	V3	6.34	5.946	6.736	81.04	61.633	106.568
(B)							
Effect	Visit		Dif. Log₂ (I)–(II)	CI95% Dif.		t-value	p-value
	(I)	(II)		Inf.	Sup.		
Visit	V0	V1	0.47	−0.22	1.157	1.7807	0.2891
		V2	−0.55	−1.235	0.142	−2.0762	0.1685
		V3	0.46	−0.226	1.151	1.759	0.2997
	V1	V2	−1.02	−1.704	−0.327	−3.857	0.0012
		V3	−0.01	−0.694	0.683	−0.0218	1
	V2	V3	1.01	0.321	1.698	3.8352	0.0013

Note: The bold text represents statistically significant values.

Abbreviations: sIgA, secretory IgA µg/mL; Log₂, log values of sIgA; CI, confidence interval; Est, statistic value.

Table 3 (A) Average Levels of sTNFαRII at All Visits; **(B)** Difference Between Averages of Significant Effects in Levels of sTNFαRII at All Visits

(A)							
Effect		Log2			pg/mL		
		Est.	CI95% Est.		Est.	CI95% for est.	
			Inf.	Sup.		Inf.	Sup.
Visit	V0	6.39	5.971	6.816	84.08	62.746	112.68
	V1	5.78	5.354	6.199	54.82	40.909	73.464
	V2	6.48	6.059	6.904	89.35	66.672	119.73
	V3	5.94	5.515	6.36	61.28	45.726	82.115
(B)							
Effect	Visit		Dif. Log₂ (I)–(II)	CI95% Dif.		t-value	p-value
	(I)	(II)		Inf.	Sup.		
Visit	V0	V1	0.62	0.203	1.032	3.8949	0.001
		V2	−0.09	−0.502	0.327	−0.5526	0.9456
		V3	0.46	0.042	0.871	2.8812	0.0249
	V1	V2	−0.7	−1.119	−0.29	−4.4475	0.0001
		V3	−0.16	−0.575	0.254	−1.0137	0.7419
	V2	V3	0.54	0.13	0.959	3.4338	0.0049

Note: The bold text represents statistically significant values.

Abbreviations: sTNFαRII, soluble fraction of receptor II of tumor necrosis factor α pg/mL; Log₂, log values of sTNFαRII; CI, Confidence Interval; Est, statistic value.

Table 4 (A) Average Levels of Cortisol at All Visits; **(B)** Difference Between Averages of Significant Effects in Levels of Cortisol at All Visits

(A)							
Effect		Log2			ng/mL		
		Est.	CI95% Est.		Est.	CI95% for Est.	
			Inf.	Sup.		Inf.	Sup.
Visit	V0	2.01	1.773	2.24	4.02	3.418	4.725
	V1	2.61	2.375	2.842	6.1	5.186	7.169
	V2	2.38	2.145	2.612	5.2	4.424	6.115
	V3	2.12	1.883	2.35	4.34	3.689	5.099
(B)							
Effect	Visit		Dif. Log₂ (I)–(II)	CI95% Dif.		t-value	p-value
	(I)	(II)		Inf.	Sup.		
Visit	V0	V1	−0.6	−0.895	−0.308	−5.3534	<0.0001
		V2	−0.37	−0.666	−0.078	−3.3133	0.0071
		V3	−0.11	−0.404	0.184	−0.9805	0.7609
	V1	V2	0.23	−0.065	0.523	2.0401	0.1809
		V3	0.49	0.197	0.785	4.3729	0.0002
	V2	V3	0.26	−0.032	0.556	2.3328	0.098

Note: The bold text represents statistically significant values.

Abbreviations: cortisol, ng/mL; Log₂, log values of cortisol; CI, confidence interval; Est: statistic value.

The evolution of each biomarker was assessed. Table 2 shows the sIgA levels at all visits. The highest levels are observed at V2. The level of sIgA at V2 was significantly higher than at V1 ($p=0.001$) and V3 ($p=0.001$). At V2, the sIgA level was approximately 2 times the level detected at V1 and 2 times that detected at V3 (Table 2A and B).

The level of sTNF α R2 at V2 was significantly higher than at V1 ($p<0.001$) and V3 ($p=0.005$). The V2 level was approximately 1.62 times the V1 level and 1.45 times the level detected at V3 (Table 3A and B).

The highest levels of cortisol are observed at V1 and the levels decrease to levels close to the baseline until V3. The level of Cortisol at V0 was significantly lower than at V1 and V2. The V1 level was approximately 1.5 times the V0 level and the V2 level 1.3 times that detected at V0 (Table 4A and B).

The differences of sAA levels were not statistically significant at any visit. But it should be mentioned that, with a significant level <0.1 , the level of Alpha amylase at V0 was significantly higher than at V2 (1.3 times) (Table 5A and B).

Testosterone did not show statistically significant differences at any of the visits. Also, worthy of highlight, with a level of 0.01, the testosterone level at V1 is significantly (approximately 1.1 times) higher than at V2 (Table 6A and B).

Table 5 (A) Average Levels of sAA at All Visits (Average); **(B)** Difference Between Averages of Significant Effects in Levels of sAA at All Visits

(A)							
Effect		Log2			U/mL		
		Est.	CI95% Est.		Est.	CI95% for Est.	
			Inf.	Sup.		Inf.	Sup.
Visit	V0	6.22	5.909	6.523	74.32	60.068	91.959
	V1	6	5.695	6.31	64.1	51.81	79.317
	V2	5.88	5.571	6.185	58.81	47.531	72.766
	V3	5.93	5.623	6.237	60.96	49.272	75.43
(B)							
Effect	Visit		Dif. Log₂ (I)–(II)	CI95% Dif.		t-value	p-value
	(I)	(II)		Inf.	Sup.		
Visit	V0	V1	0.2	−0.181	0.572	1.3571	0.5293
		V2	0.35	−0.029	0.724	2.4122	0.0818
		V3	0.29	−0.085	0.668	2.0246	0.1864
	V1	V2	0.15	−0.225	0.528	1.0551	0.7175
		V3	0.1	−0.281	0.473	0.6675	0.9091
	V2	V3	−0.06	−0.432	0.321	−0.3876	0.9801

Abbreviations: sAA, α -amylase U/mL; Log₂, log values of sAA; CI, confidence interval; Est, statistic value.

Table 6 (A) Average Levels of Testosterone at All Visits; **(B)** Difference Between Averages of Significant Effects in Levels of Testosterone at All Visits

(A)							
Effect		Log2			pg/mL		
		Est.	CI95% Est.		Est.	CI95% for Est.	
			Inf.	Sup.		Inf.	Sup.
Visit	V0	6.01	5.794	6.228	64.49	55.497	74.942
	V1	6.16	5.94	6.373	71.32	61.376	82.882
	V2	6.01	5.788	6.222	64.23	55.268	74.634
	V3	6.04	5.825	6.258	65.88	56.691	76.555
(B)							
Effect	Visit		Dif. Log ₂ (I)–(II)	CI95% Dif.		t-value	p-value
	(I)	(II)		Inf.	Sup.		
Visit	V0	V1	−0.15	−0.314	0.023	−2.2589	0.1153
		V2	0.01	−0.162	0.174	0.0926	0.9997
		V3	−0.03	−0.199	0.138	−0.4775	0.9639
	V1	V2	0.15	−0.017	0.319	2.3515	0.094
		V3	0.11	−0.054	0.283	1.7814	0.2888
	V2	V3	−0.04	−0.205	0.132	−0.57	0.9407

Note: The italicized text represents statistically significant values with a level of 0.01.

Abbreviations: Testosterone, pg/mL; Log₂, log values of sAA; CI, confidence interval; Est, statistic value.

Relationship of Biomarkers with Demographic Variables

The relationship between cortisol level and age was statistically significant ($p=0.043$) at Visit 0. It was a negative relationship: as age increased, the level of cortisol was lower (Figure 2). With a significant level <0.1 , the relationships of age with sAA and sIgA would also be significant

($p=0.056$ and $p=0.053$, respectively). In the first case, the relationship was positive, with higher levels among older individuals, and in the second case, the relationship was negative. It should be highlighted that the relationship between sAA and age was statistically significant at Visit 1 ($p=0.041$). At all visits, the level of testosterone was statistically higher in men than in women (Table 7).

Relationship of Biomarkers with Subjective Scales

VAS variations were not statistically significant with any of the biomarkers except for the sIgA, but only at V0 ($p=0.024$). sIgA levels were higher in subjects with higher scores on the VAS scale (Figure 3). At V2, sIgA remained at the limit of significance. Two individuals were considered as outliers and eliminated. Then, the model fulfilled all the necessary hypotheses and the relationship with the VAS scale was significant.

Discussion

Our results confirm previous studies showing that ASA produces severe pain in postoperative period, reaching a moderate-high intensity (VAS value of 6) with a peak of pain located between 24 and 36 hrs.^{18,29,30} Although the VAS has been universally validated for the measurement of pain, it has obvious limitations.³¹ Thus, there is a general consensus that new objective measures of pain are required, including salivary biomarkers.^{5,32}

Our group had analyzed the inter-individual differences and intersession variability in the saliva of healthy subjects for the current selected biomarkers.¹⁵ We found significant variance in male and female testosterone, indicating that this hormone seems to be a poor salivary biomarker for pain. However, due to recent findings by Choi on the

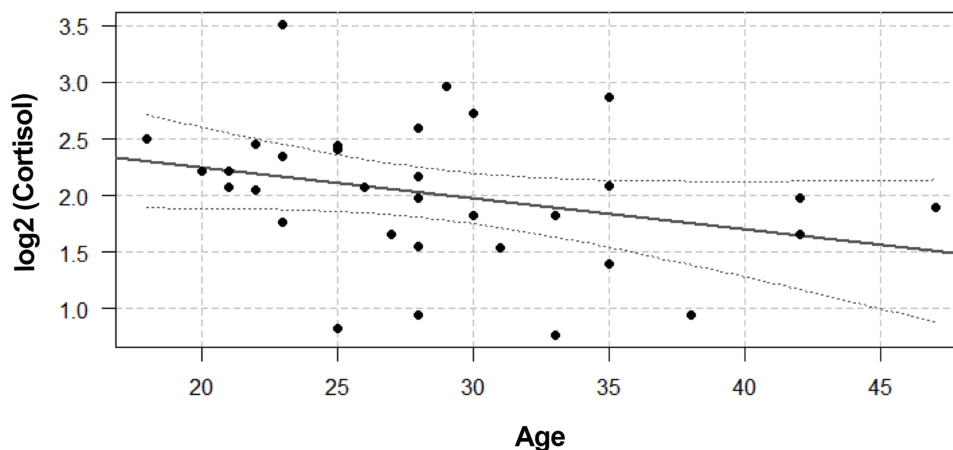


Figure 2 Spearman correlation plot and 95% CI showing a negative correlation between cortisol level and age.

Table 7 Testosterone Levels in Males and in Females at All Visits

Visit	Gender	Mean	SD	P-value
V0	M	82.33	30.96	0.0034
	F	53.84	22.75	
V1	M	88.73	28.46	0.0005
	F	57.86	18.05	
V2	M	83.46	34.14	0.0017
	F	51.87	19.62	
V3	M	86.87	28.51	0.0001
	F	49.79	17.76	

Notes: N= 19 males, 13 females. V0, baseline; V1, pre-surgery; V2, 1 hr post-surgery, and V3, 72 hrs post-surgery.

Abbreviation: SD, standard deviation.

importance of considering the effects of testosterone on pain, we considered it necessary to include testosterone in this study. Choi reported that lower levels of testosterone allow greater tolerance to pain, an important aspect to consider when treating patients for pain.¹²

The results showed that sIgA is the biomarker that presents the highest correlation with pain. sIgA is one of the main antibodies in the oral mucosa³³ and it plays an essential role in maintaining the integrity of the mucous membrane.³⁴ Its levels on the surface of oral mucosa have been shown to be related with the regulation of the development of inflammatory responses.³⁴

Our results are consistent with previous reports, where patients affected by oral lichen planus, a chronic disease, presented higher values for sIgA than control subjects.³⁵ However, da Silva and colleagues found a negative correlation between oral pain intensity and sIgA levels in children.⁸

sTNF α RII is the other biomarker whose concentration is raised at V2. Goodin et al showed that sTNF α RII in oral fluids significantly decreased from baseline in relation to all three experimental pain modalities.⁴ Contrary to that study, our results reported a significant increase, which correlates with the highest values on the VAS.

Although there is a study that considered that sAA measurements could be a good biomarker of pain,¹¹ our results did not find statistically significant differences between visits. These results are consistent with a previous study carried out by our group analyzing the reliability of this potential pain biomarker in the saliva of healthy subjects. That study did not find acceptable levels of reproducibility between two measurements.¹⁵

Cortisol response to pain does not appear to be purely a function of the pain experience, because cortisol at V0 was significantly lower than at V1 and V2. It may be that other stress-related features, such as sympathetic activation, may be involved in the cortisol elevation detected.^{4,36}

Conclusion

In contrast to other studies, in this study, all the biomarkers were analyzed together, relating them to the VAS and in the postoperative period of a surgery in which the evolution of pain is well described. This is the first study using this “clinical model” of acute postoperative pain, which in our opinion has some advantages. Patients who are going to undergo ASA surgery are healthy patients, without pain prior to surgery.

Regarding the increase of cortisol concentration at the pre-surgery visit, we believe that it is related to the presence of anxiety and depression as detected by the HADS scale. It

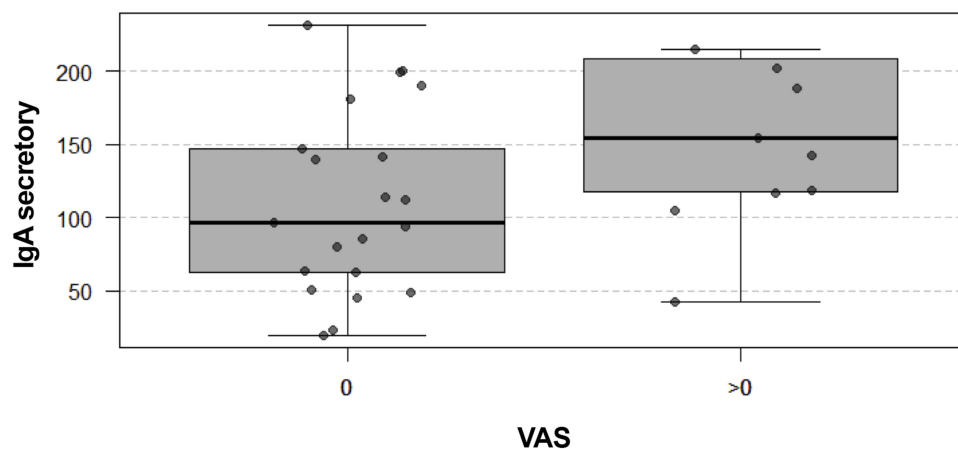


Figure 3 The graph presents the relationship between VAS and sIgA at V0. VAS is considered as a discrete variable with two levels: 0 and >0 (up to a maximum of 2). IgA levels are significantly higher in the group of individuals that score higher than 0 on the VAS scale. The VAS is statistically significant at IgA levels.

can be concluded that cortisol, under these circumstances, could be a biomarker more related to stress than to pain.

In this study, sIgA and sTNF α RII are the two potential biomarkers that present correlation with the VAS and these salivary substances showed acceptable levels of reproducibility in healthy subjects.¹⁵ Nevertheless, further studies are required, including studies with other types of post-surgical pain before proposing them as valid pain biomarkers in future clinical studies.

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Disclosure

The authors report no conflicts of interest in this work.

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