



HAL
open science

Potential applications of lipid peroxidation products -F4 -neuroprostanes, F3 -neuroprostanes n-6 DPA , F2 -dihomo-isoprostanes and F2 -isoprostanes -in the evaluation of the allograft function in renal transplantation

Ignacio de Las Heras-Gómez, Sonia Medina, Teresa Casas-Pina, Lidia Marín-Soler, Anna Tomás, Pedro Martínez-Hernández, Camille Oger, Jean-Marie Galano, Thierry Durand, Luisa Jimeno, et al.

► To cite this version:

Ignacio de Las Heras-Gómez, Sonia Medina, Teresa Casas-Pina, Lidia Marín-Soler, Anna Tomás, et al.. Potential applications of lipid peroxidation products -F4 -neuroprostanes, F3 -neuroprostanes n-6 DPA , F2 -dihomo-isoprostanes and F2 -isoprostanes -in the evaluation of the allograft function in renal transplantation. Free Radical Biology and Medicine, 2017, 10.1016/j.freeradbiomed.2017.01.019 . hal-02593586

HAL Id: hal-02593586

<https://hal.science/hal-02593586>

Submitted on 15 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Potential applications of lipid peroxidation products – F₄-neuroprostanes, F₃-neuroprostanes_{n-6 DPA}, F₂-dihomo-isoprostanes and F₂-isoprostanes - in the evaluation of the allograft function in renal transplantation

Ignacio De las Heras-Gómez^a, Sonia Medina^b, Teresa Casas-Pina^{a,*}, Lidia Marín-Soler^b, Anna Tomás^b, Pedro Martínez-Hernández^a, Camille Oger^d, Jean-Marie Galano^d, Thierry Durand^d, Luisa Jimeno^c, Santiago Llorente^c, Elena Lozoya^a, Federico Ferreres^b, Ángel Gil-Izquierdo^{b,*}

^a Clinical Analysis Service, University Hospital Virgen de la Arrixaca, Ctra. Madrid-Cartagena, s/n, 30120, El Palmar, Murcia, Spain

^b Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS (CSIC), P.O. Box 164, 30100 Campus University Espinardo, Murcia, Spain

^c Nephrology Service, University Hospital Virgen de la Arrixaca, Ctra. Madrid-Cartagena, s/n, 30120, El Palmar, Murcia, Spain

^d Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 - CNRS - University of Montpellier - ENSCM, Faculty of Pharmacy, Montpellier, France

A B S T R A C T

F₄-neuroprostanes, F₃-neuroprostanes_{n-6 DPA}, and F₂-dihomo-isoprostanes, metabolites of non-enzymatic lipid peroxidation of polyunsaturated fatty acids [docosahexaenoic acid, n-6 docosapentanoic acid, and adrenic acid respectively], have become important biomarkers for oxidative stress in several diseases like epilepsy and alzheimer. These biomarkers and the 15-F_{2t}-isoprostane (also known as 8-iso-PGF_{2α}), a F₂-isoprostane isomer measured as reference oxidative marker at systemic level, were analyzed by UHPLC-QqQ-MS/MS in the urine of 60 renal recipients from cadaveric donors of the Nephrology Unit of the University Hospital Virgen de la Arrixaca, at six different times during the first six months after renal transplantation, and were compared with a control group of 60 healthy subjects from the same hospital. A total of 11 metabolites were analyzed and different patterns were observed. A tendency to decrease was observed in three metabolites (4-*epi*-4-F_{3t}-NeuroP_{n-6 DPA}, *ent*-7(*RS*)-7-F_{2t}-dihomo-IsoP, and *ent*-7(*S*)-7-F_{2t}-dihomo-IsoP) and in our reference oxidative marker (15-F_{2t}-IsoP) when kidney function improved and the excretion of urine proteins decreased. These results suggest that these three biomarkers of oxidative stress could be useful to assess renal function in the posttransplant phase. Unfortunately, little is known about this kind of biomarker in this cohort of patients, so further investigation would be required in the clinical field to clarify the relationship between oxidative stress and the graft function, as well as the usefulness of these biomarkers as rejection markers.

1. Introduction

Kidney transplantation is considered the best available treatment for patients with end-stage renal disease. In recent decades, there has been an increased demand for this kind of transplantation. This can be attributed to the increasing number of patients with this pathology - which may result from chronic diseases such as metabolic disorders, diabetes mellitus, and hypertension [1], as a result of the new lifestyle acquired in western countries.

Although short-term renal allograft survival has recently increased

due to new immunosuppressive therapies, allograft renal rejection is still one of the most important complications after renal transplantation, directly leads to allograft loss, and is detrimental to long-term survival. Graft rejection is a complex pathophysiological process that requires analyses at different levels for its complete understanding [2] and undermines the full benefits of the transplant [3]. Consequently, timely detection and treatment of rejection is an important goal in the posttransplant surveillance.

The standard care with serum creatinine (CrS) measurements and biopsy upon allograft dysfunction implies that rejection is detected at

Abbreviations: CrS, Serum creatinine; OS, Oxidative stress; ROS, Reactive oxygen species; UHPLC-QqQ-MS/MS, Ultra High Pressure Liquid Chromatography-triple quadrupole-Tandem Mass Spectrometry; F₃-NeuroPs_{n-6 DPA}, F₃-Neuroprostanes_{n-6 DPA}; F₄-NeuroPs, F₄-Neuroprostanes; F₂-dihomo-IsoPs, F₂-dihomo-isoprostanes; n-6 DPA, n-6 docosapentanoic acid; DHA, Docosahexaenoic acid; AdA, Adrenic acid; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane (8-iso-PGF_{2α}); eGFR, Estimated Glomerular Renal Filtration; BUN, Blood Urea Nitrogen; Albs, Serum Albumin; Hct, Hematocrit levels; Prot/Creat ratio, Protein/creatinine ratio; ISE, Internal standard element; BTR, Below test range; LOD/LOQ, Limit of detection and/or quantification

* Corresponding author.

E-mail addresses: tcasaspina@gmail.com (T. Casas-Pina), angelgil@cebas.csic.es (Á. Gil-Izquierdo).

an advanced stage. CrS concentrations are dependent upon age, gender, muscle mass, medication, and hydration status and may not be altered until a significant amount of kidney function is lost [4]. Therefore, the measurement of only the CrS as the gold standard marker of kidney function carries the risk of missing an important therapeutic window because of the time lag between the inciting insult and the diagnostic elevation of creatinine. On the other hand, biopsy is an invasive procedure and, although it has become safer and its interpretation more standardized [5], bleeding and subsequent graft loss still occur, while sampling errors and interobserver variability in biopsy reading remain problematic.

Metabolomics, a relatively new tool for the investigation of metabolic changes, is concerned with the high throughput measurement of all the small-molecule metabolites in the metabolome of a system [6]. This capacity to measure hundreds of thousands of important metabolites quickly has opened the door to many potential applications in a large number of areas, including plant research [7], nutrition [8], pharmaceutical research [9], cardiovascular disease [10], and organ transplantation [11].

Metabolites measurements have been part of organ transplant monitoring for more than 60 years [12]. While most measurements have been restricted to just a few well known compounds - such as creatinine, glucose, and urea - there is a large body of lesser known metabolites involved in pathophysiological process which remain to be discovered.

Oxidative stress (OS) is a biochemical state in which reactive oxygen species (ROS) are generated and it has been associated with several pathological states, including renal transplantation [13]. The ROS are extremely unstable and highly reactive metabolites, which makes them difficult to detect *in vivo*. Oxidative damage in humans can be measured as the oxidation of different biomolecules - such as lipids, proteins, or nucleic acids - using metabolomics tools. In particular, lipidomics (targeted metabolomics) might be used with the hope of both identifying a biomarker of lipid oxidation that could reflect kidney function and, in the future, seeking its involvement in rejections, thereby trying to avoid the high risk associated with the graft biopsy.

Using a Ultra High Pressure Liquid Chromatography-triple quadrupole-Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS), we measured F₃-neuroprostanes *n*-6 DPA (F₃-NeuroPs *n*-6 DPA), F₄-neuroprostanes (F₄-NeuroPs), and F₂-dihomo-isoprostanes (F₂-dihomo-IsoPs), biomarkers of the non-enzymatic lipid peroxidation of *n*-6 docosapentaenoic acid (*n*-6 DPA), docosahexaenoic acid (DHA), and adrenic acid (AdA), respectively. Furthermore we measured the 15-F_{2t}-isoprostane (15-F_{2t}-IsoP or also known as 8-iso-PGF_{2α}), derived from arachidonic acid, as reference oxidative marker at systemic level.

Thus, the main aim of our study was to evaluate the changes in the evolution of these metabolites of lipid peroxidation during the short-term posttransplantation period, in comparison with a healthy group, in order to identify potential biomarkers of prognosis and their applications in the evaluation of graft function after transplantation.

2. Subjects and methods

2.1. Selection of study participants

In a prospective longitudinal study between October 2013 and May 2015, we followed up 60 kidney transplant recipients from the Nephrology Unit of the University Hospital Virgen de la Arrixaca, aged between 16 and 72 years old. Sixteen of them were excluded from the final data analysis since the technicians could not collect blood and urine samples in a timely manner because of missed clinic appointments, two of them died, and another one had an acute cellular rejection with allograft loss, leaving a final study population of 41 patients. Forty-eight percent of them suffered delayed kidney function after the transplant and 13 were biopsied because of a worsening renal function. Those patients with acute illnesses or a previous renal

transplant were excluded from the study. All the transplanted kidneys were from cadaveric donors: 46 died from cerebrovascular accidents and 14 from traumatic brain injury. Every patient selected received anticalcineurinic drugs, steroids, and mycophenolate mofetyl as immunosuppressive therapy. In addition, we recruited 60 healthy subjects from the same hospital, aged between 24 and 67 years old. All patients gave written informed consent for the experiment and the study was approved by the Bioethics Committee of the University Hospital Virgen de la Arrixaca (Murcia, Spain), and the research was carried out in compliance with the Declaration of Helsinki [14].

2.2. Blood samples

Blood samples were collected from the patients group at six different times after transplantation: five days post transplant, ten days post transplant, and at clinic visits at one month post transplant, two months post transplant, three months post transplant, and six months post transplant, according to the renal-transplant clinic schedules. From the control group, only one blood sample was collected. Venous blood was drawn into Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) containing ethyldiaminetetraacetic acid, for whole blood, and into serum separator tubes for serum.

Hematological parameters were immediately determined using an XE-5100 automatic analyzer (Sysmex, Kobe, Japan). The serum in the tubes was allowed to clot at room temperature before centrifugation. The tubes were centrifuged at 4 °C, at 2500 rpm, for 10 min. Biochemical parameters were measured in the serum by colorimetric and turbidimetric assays, in a Cobas 8100 (Roche Diagnostics, Mannheim, Germany), performed according to the manufacturer's instructions [15]. For the evaluation of renal function we used: CrS, estimated Glomerular Renal Filtration (eGFR) by the MDRD equation, and Blood Urea Nitrogen (BUN). Moreover, the Serum Albumin (AlbS) and Hematocrit levels (Hct) were considered for the evaluation of nutritional status.

2.3. Urine sampling

First-morning urine samples were collected from the patients group at the six different and consecutive posttransplant moments. From the control group, only one first-morning urine sample was collected. They were collected in sterile, dark polystyrene pots with screw caps. The urine samples from all patients and the control group were centrifuged, aliquoted into Eppendorf tubes, and stored at -80 °C. To standardize the results, we measured first-morning creatinine and protein levels by colorimetry in a Cobas 8100 (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions [15], and we calculated the protein/creatinine ratio (Prot/Creat ratio). The urinary excretion of F₄-NeuroPs, F₃-NeuroPs_{*n*-6 DPA}, and F₂-dihomo-IsoPs was analyzed using the method described below. This method pointed out the importance of enzymatic hydrolysis of the urine samples, since F₄-NeuroPs, F₃-NeuroPs_{*n*-6 DPA}, and F₂-dihomo-IsoPs are excreted in urine as glucuronide and sulfate conjugates [16]. On the other hand, we measured the concentration of 15-F_{2t}-IsoP, in order to compare its evolution with F₄-neuroprostanes, F₃-neuroprostanes_{*n*-6 DPA} and F₂-dihomo-isoprostanes. This F₂-isoprostane isomer is the most frequently metabolite measured in the clinical assays, for this reason, it used as the oxylipin reference compound for a lot of types of pathophysiological disorders.

2.4. Chemicals and reagents

Nine NeuroPs - 4-*epi*-4-F_{3t}-NeuroP_{*n*-6 DPA}; 4-F_{3t}-NeuroP_{*n*-6 DPA}; 4-(RS)-F_{4t}-NeuroP; 4-F_{4t}-NeuroP; 10-*epi*-10-F_{4t}-NeuroP; 10-F_{4t}-NeuroP; d₄-4-(RS)-F_{4t}-NeuroP (Internal standard element (ISE 1); d₄-10-*epi*-10-F_{4t}-NeuroP (ISE 2); and d₄-10-F_{4t}-NeuroP (ISE 3) - as well as four F₂-dihomo-IsoPs - 17-*epi*-17-F_{2t}-dihomo-IsoP; 17-F_{2t}-dihomo-

IsoP; *ent*-7(*RS*)-7- F_{2t} -dihomo-IsoP; and *ent*-7(*S*)-7- F_{2t} -dihomo-IsoP - were synthesized by Durand's team at the Institute des Biomolécules Max Mosseron (IBMM) (Montpellier, France) [7–19]. The 15- F_{2t} -IsoP (8-iso-PGF_{2 α}) were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). The β -glucuronidase, type H2, from *Helix pomatia* and BIS-TRIS (Bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane) used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). All LC-MS grade solvents were obtained from J. T. Baker (Phillipsburg, NJ, USA). Chlorhydric acid was purchased from Panreac (Castellar del Vallés, Barcelona, Spain) and the Strata X-AW, 100 mg.3 mL⁻¹ solid phase extraction cartridges from Phenomenex (Torrance, CA, USA).

2.5. UHPLC-QqQ-MS/MS analysis of neuroprostanes and F_2 -dihomo-isoprostanes

The separation and quantification of the F_4 -NeuroPs, F_3 -NeuroPs_{*n*-6 DPA}, and F_2 -dihomo-IsoPs in the urine were performed using a UHPLC coupled with a 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), and the analytical method previously described [16]. The chromatographic separation was carried out on an ACQUITY BEH C₁₈ column (2.1 × 50 mm, 1.7 μ m pore size) (Waters, MA, USA). The mass spectrometry analysis was performed by multiple reaction monitoring in the negative ionization mode. The mobile phases were solvent A (Milli-Q water/acetic acid, 99.99:0.01, v/v) and solvent B (methanol/acetic acid, 99.99:0.01, v/v). The flow rate was 0.2 mL min⁻¹ using a linear gradient scheme: (t; %B): (0.0; 60.00), (7.00; 70.00), (7.01; 90.00), (10.00; 90.00), (10.01; 60.00). The electrospray ionization conditions and ion optics were those previously described [16]. The separation and quantification of 15- F_{2t} -IsoP was performed by the analytical method previously described [20]. Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies, Waldbronn, Germany). The metabolites concentrations were calculated from standard curves freshly prepared each day.

2.6. Statistical analyses

Quantitative data are presented as the median and interquartile range for non-normally distributed data, and as the mean and standard deviation for normally distributed data. Each metabolite measured was analyzed after normalization with urinary creatinine and expressed as ng mg creatinine⁻¹. The analyses of the different variables at the different posttransplant moments were performed using the Wilcoxon test. The comparison of groups at each evaluation was performed using Kruskal-Wallis and Mann-Whitney tests. The correlations among the study variables were performed by the Spearman rank-order correlation. For continuous variables, univariate comparisons of the study population and healthy controls were carried out using the Mann-Whitney *U* test. The statistical analyses were performed using the SPSS 15.0 software package (LEAD Technologies Inc., Chicago, USA) and the level of statistical significance was set at $P < 0.05$.

3. Results

3.1. Biochemical parameters evolution

In Table 1 the values of the different parameters measured at the six posttransplant moments, for the evaluation of kidney function and nutritional status, are shown.

In this study, we compared the biochemical parameters measured for the evaluation of allograft function at each moment with the consecutive one and we expected changes in their evolution. Thus, the serum levels of CrS and MDRD improved significantly between 5 and 10 days post transplant and between 10 days and 1 month post transplant ($P < 0.05$ for all), with a maximum for MDRD and a

minimum for CrS at 6 months post transplant. Although BUN decreased progressively at the six different moments after transplantation, we only found a significant difference between 10 days and 1 month post transplant ($P < 0.001$). The reduction between the others consecutive moment was not statistically significant ($P > 0.05$). The Prot/Creat ratio in urine decreased between 5 days and 3 months post transplant, with significant differences between 5 and 10 days post transplant and 10 days and 1 month post transplant ($P < 0.05$ for all), and with a minimum at 3 months post transplant. In addition we observed a significant increases for AlbS and Hct between 10 days and 1 month post transplant, and between 1 and 2 months post transplant ($P < 0.05$ for all), with a maximum for both at 6 months post transplant.

When we compared the study variables between 5 days and 6 months post transplant (corresponding to the worst and best eGFR, respectively), we observed significantly differences in all the biochemical parameters measured ($P < 0.001$ for all).

3.2. Metabolites in renal recipients and healthy subjects

Concerning the qualitative profiles of the OS biomarkers of the healthy and renal transplant patients of this study, NeuroPs were mainly detected in renal recipients and not in healthy subjects, because they were present at very low levels, below the limit of detection and/or quantification (LOD/LOQ). In the control group, we only detected one NeuroP, 4- F_{3t} -NeuroP_{*n*-6 DPA}, but we didn't detect its 4-epimer, 4-*epi*-4- F_{3t} -NeuroP_{*n*-6 DPA}, maybe due to the higher LOD/LOQ that we obtained in our assay [16]. The F_2 -dihomo-isoprostanes were detected in patients and healthy subjects. So, we compared the study variables in the 41 renal recipients between five days and 6 months post transplant, (corresponding to the worst and best eGFR, respectively), and with those of healthy subjects. When a comparison was developed at five days post transplant with the control group, the patients showed significantly higher levels of *ent*-7(*RS*)-7- F_{2t} -dihomo-IsoP ($P < 0.05$) and significantly lower levels of 4- F_{3t} -NeuroP_{*n*-6 DPA} ($P < 0.05$), 17-*epi*-17- F_{2t} -dihomo-IsoP ($P < 0.01$), 17- F_{2t} -dihomo-IsoP ($P < 0.001$), and *ent*-7(*S*)-7- F_{2t} -dihomo-IsoP ($P < 0.001$). At 6 months post transplant, significantly lower levels of 4- F_{3t} -NeuroP_{*n*-6 DPA} ($P < 0.05$), 17-*epi*-17- F_{2t} -dihomo-IsoP ($P < 0.05$), 17- F_{2t} -dihomo-IsoP ($P < 0.05$), and *ent*-7(*S*)-7- F_{2t} -dihomo-IsoP ($P < 0.001$) were detected in the patients. Furthermore, *ent*-7(*RS*)-7- F_{2t} -dihomo-IsoP did not differ significantly between renal recipients and healthy subjects at 6 months posttransplant (Fig. 1).

3.3. Qualitative and quantitative profiles of F_4 -NeuroPs, F_3 -NeuroPs_{*n*-6 DPA}, and F_2 -dihomo-isoprostanes in the transplant recipients

Eleven metabolites were analyzed in the urine of the volunteers. Their identification was carried out according to their molecular mass, tandem mass spectrometry fragmentation pattern, and retention time.

For three of the F_4 -NeuroPs from DHA measured, 4(*RS*)- F_{4t} -NeuroP, 4- F_{4t} -NeuroP, and 10- F_{4t} -NeuroP, we observed oscillations at the different moments and no correlation with renal function. On the other hand, 10-*epi*-10- F_{4t} -NeuroP, the other F_4 -NeuroP derived from this fatty acid, increased over the posttransplant stage. However, there were no statistically significant differences when comparing the concentrations of 10-*epi*-10- F_{4t} -NeuroP at the different consecutive moments ($P > 0.05$ for all the comparisons) or when comparing day 5 and month 6 post transplant ($P = 0.182$) (Fig. 2).

The F_3 -NeuroPs_{*n*-6 DPA}, metabolites derived from *n*-6 DPA, differed in their evolution over the six posttransplant moments. For example, 4-*epi*-4- F_{3t} -NeuroP_{*n*-6 DPA} decreased in a concomitant manner with the improvement of the kidney function, with a minimum at 6 months post transplant - which showed the best glomerular filtration and the minimum Prot/Creat ratio. Despite this progressive decline, we did not find statistically significant differences when we compared the concentrations of this NeuroP between different consecutive moments

Table 1
Evolution of biochemical parameters for six moments after transplantation.

	Five days posttransplant	Ten days posttransplant	One month posttransplant	Two months posttransplant	Three months posttransplant	Six months posttransplant	Healthy subjects
CrS (mg dL ⁻¹)	4.75 (2.40–6.03)	3.29 (2.01–5.33)	1.76 (1.64–2.22)	1.67 (1.55–1.97)	1.72 (1.56–2.07)	1.61 (1.42–2.06)	0.81 (0.73–0.93)
MDRD(mL/min/1,73 m ²)	13.71 (9.01–30.42)	19.81 (10.12–34.71)	41.04 (31.51–48.92)	40.54 (35.91–51.79)	41.42 (33.94–51.56)	45.91 (34.52–55.50)	88.50 (69.31–112.12)
BUN (mg dL ⁻¹)	141.00 (88.50–182.50)	168.00 (96.02–214.58)	71.50 (54.57–103.33)	66.54 (50.71–85.02)	68.07 (52.04–99.56)	61.01 (46.02–85.55)	31.00 (23.50–39.56)
Prot/creat ratio (mg g ⁻¹ creat)	827.42 (507.03–2441.05)	295.22 (205.25–943.02)	167.81 (128.03–248.52)	156.81 (95.12–312.41)	146.46 (84.58–280.16)	146.54 (81.56–279.62)	BTR
Albs (g dL ⁻¹)	3.40 (3.00–3.80)	3.50 (3.20–3.78)	4.10 (3.65–4.45)	4.25 (3.98–4.53)	4.40 (4.15–4.60)	4.50 (4.10–4.63)	4.54 (4.12–4.96)
Hct (%)	30.82 (28.01–34.52)	29.92 (27.21–34.36)	34.34 (30.82–39.63)	37.77 (33.42–41.71)	40.12 (36.83–43.96)	40.74 (36.55–45.91)	42.92 (40.41–47.12)

Data are presented as median and (interquartile range). BTR (Below test range). CrS (Serum creatinine). Urea Nitrogen (BUN). Prot/creat ratio (Protein/creatinine ratio). Serum Albumin (Albs) and Hematocrit levels (Hct).

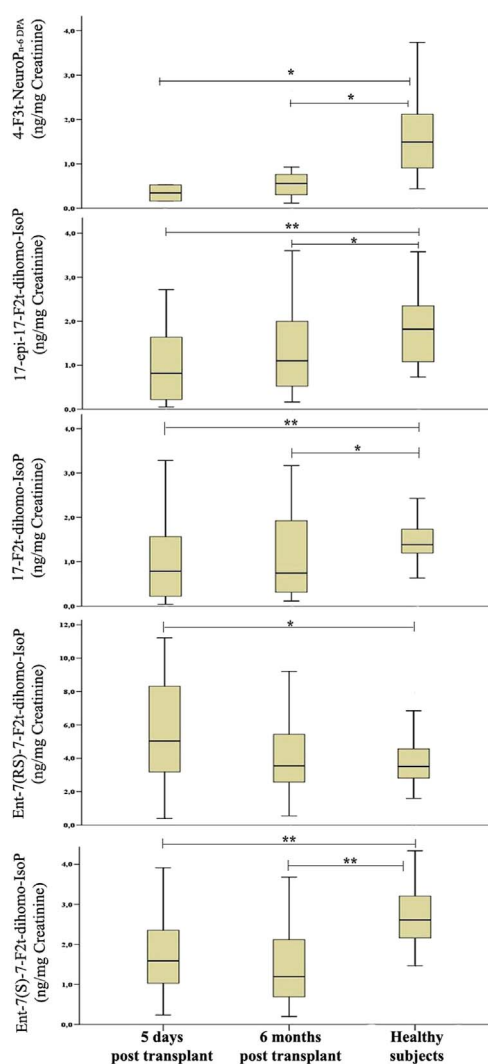


Fig. 1. Comparison at 5 days and 6 months post transplant with healthy subjects. Outlier data points were removed.

($P > 0.05$ for all the comparisons), but we found a significant decrease when we compared it at 5 days and six months post transplant ($P = 0.01$). 4-F_{3t}-NeuroP_{n-6 DPA}, the other F₃-NeuroP_{n-6 DPA} measured, did not have a defined pattern, with oscillations at the different moments and without correlation with renal function. The concentrations of this metabolite were similar at the beginning and end of the

study ($P = 0.655$) (Fig. 2).

In relation to the F₂-dihomo-IsoPs, OS biomarkers of AdA, two different patterns emerged. On the one hand, 17-*epi*-17-F_{2t}-dihomo-IsoP and 17-F_{2t}-dihomo-IsoP presented increases and decreases, with no correlation with renal function; on the other hand, we found a tendency of the amounts of *ent*-7(RS)-7-F_{2t}-dihomo-IsoP and *ent*-7(S)-7-F_{2t}-dihomo-IsoP to decrease during the posttransplant period with a minimum at 3 months post transplant. We compared their concentrations at 5 days and 6 months post transplant, and we found only a trend to decrease ($P = 0.072$ and $P = 0.162$, respectively), but when we compared their concentrations at 5 days and 3 months post transplant, corresponding to the highest and the lowest protein/creatinine ratios respectively, a significant decrease in both metabolites was observed ($P < 0.05$ for both) between these moments (Fig. 2.). Therefore, the *ent*-7(RS)-7-F_{2t}-dihomo-IsoP and *ent*-7(S)-7-F_{2t}-dihomo-IsoP concentrations decreased as renal function improved.

The 15-F_{2t}-IsoP, the F₂-isoprostane isomer measured as reference oxidative marker at systemic level, showed a tendency to decrease during the posttransplant period such as it was detected for the other metabolites. However, there was no statistically significant difference when comparing its concentrations at the different consecutive moments ($P > 0.05$ for all the comparisons). When we compared its amount at day 5 (83,3 ng mg creatinine⁻¹) and month 6 post transplant (62,6 ng mg creatinine⁻¹) only a trend to decrease ($P = 0.170$) was underlined.

When we examined the relationships between the metabolites, F₄-NeuroPs, F₃-NeuroPs_{n-6 DPA}, F₂-dihomo-IsoPs and 15-F_{2t}-IsoP, and eGFR at the six different moments after renal transplantation, no conclusive outcomes were observed.

Among the metabolites and the protein/creatinine ratio at the six different moments after renal transplantation, there were several significant correlations for 4-*epi*-4-F_{3t}-NeuroP_{n-6 DPA}: at 1 month post transplant ($r_s = 0.534$; $P < 0.02$), at 2 months post transplant ($r_s = 0.52$; $P = 0.02$), and at 6 months post transplant ($r_s = 0.51$; $P < 0.01$). At 3 months post transplant, there was a slight relationship ($r_s = 0.237$; $P < 0.123$), and at 5 and 10 days post transplant, no correlation with urinary protein excretion was detected. For *ent*-7(RS)-7-F_{2t}-dihomo-IsoP we only found a significant correlations at 1 month post transplant ($r_s = 0.533$; $P = 0.02$), and for *ent*-7(S)-7-F_{2t}-dihomo-IsoP at 2 months post transplant ($r_s = 0.362$; $P = 0.035$). For our reference metabolite, 15-F_{2t}-IsoP, we detected a significant correlation at 3 months post transplant ($r_s = 0.464$; $P < 0.05$), and at 6 months post transplant ($r_s = 0.612$; $P < 0.02$).

Furthermore, we separated the transplantation group, depending on the protein/creatinine ratio, into two groups, group A₁ for patients with a ratio < 30 mg/g and group A₂ for patients with a ratio > 30 mgg⁻¹, and we found no differences between the two groups.

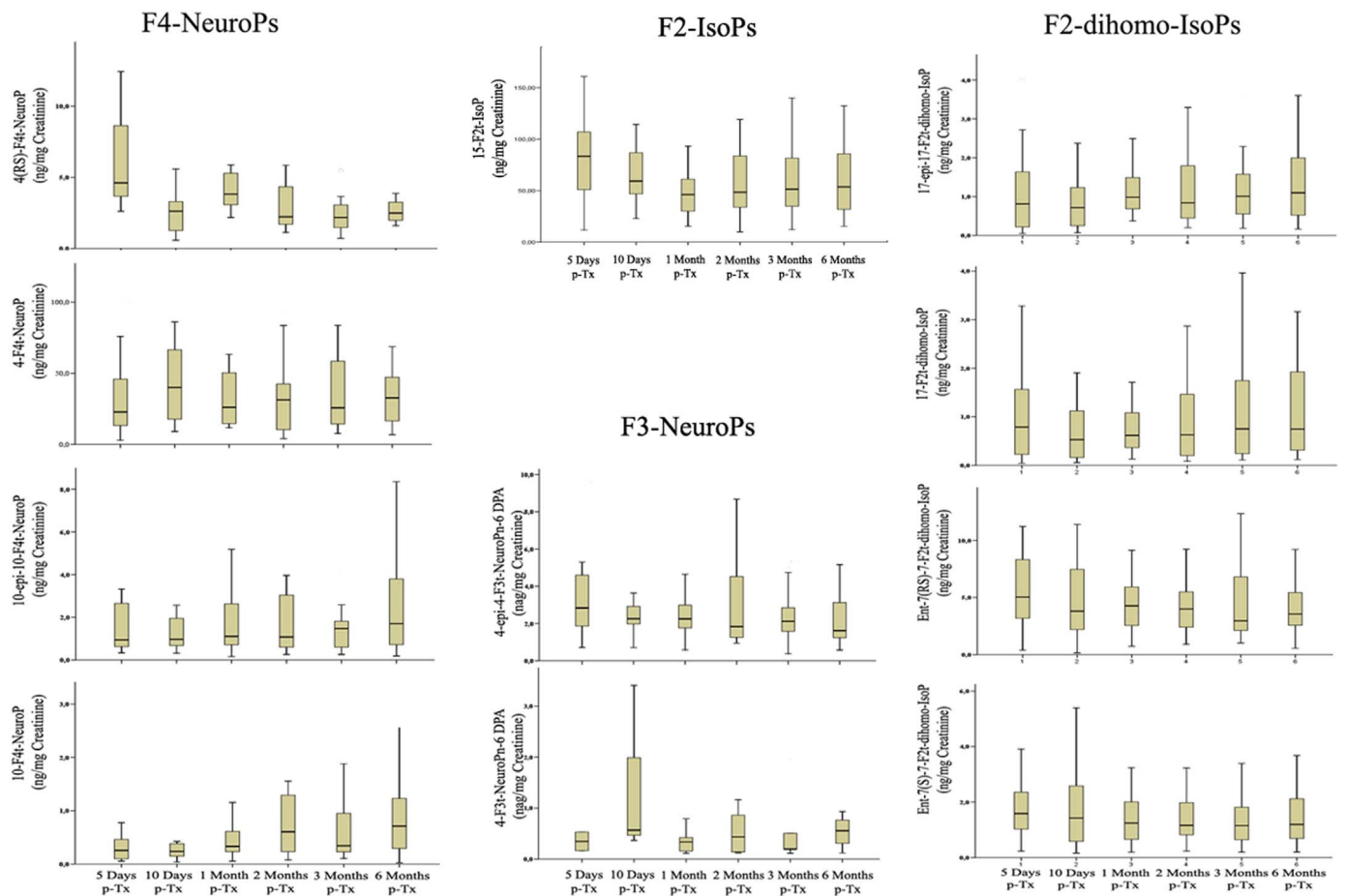


Fig. 2. F4-NeuroPs, F₂-IsoPs, F₃-NeuroPs and F₂-Di-homo-IsoPs evolution in the different posttransplant moments. Outlier data points were removed. p-Tx: posttransplant.

4. Discussion

The current lifestyle of Western societies is causing an increase in the incidence of metabolic disorders [1] that trigger long-term chronic diseases such as chronic renal failure. The best treatment of this disease is a kidney transplant, since it improves the patient's quality of life and decreases the numerous complications associated with dialysis [21]. The rejection of the transplanted organ is one of the most serious complications of this kind of treatment and the graft prognosis gets worse in the long-term, sometimes causing its complete loss. Although this complication is becoming increasingly rare, thanks to new immunosuppressants, it remains a fact among kidney transplant recipients [2].

The rejection of a solid organ is a complex pathophysiological process that goes beyond the immunological rejection and to which many factors contribute [1], including OS [13]. An oxidative imbalance, caused by an increase in the generation of ROS, and a decreased ability to restore the redox balance of the body produce negative effects in cells, worsening their function and shortening life expectancy [22].

This adverse effect of OS on cells is partly due to the destruction of fatty acids that make up the cell membrane, such as DHA, *n*-6 DPA, and AdA [23]. Three of the major targets of the lipid peroxidation process are the brain, kidney, and liver. Thus, the toxicity of lipid peroxidation products in mammals generally involves neurotoxicity, nephrotoxicity, and hepatotoxicity [24]. Additionally, the kidney is very vulnerable to ROS damage because renal lipids are composed of an abundance of long-chain polyunsaturated fatty acids. The kidneys are a site of both the production and clearance of these metabolites, so the best way to explore their production in kidney diseases is through urine samples. Some recent studies related OS to kidney damage [25], but few of them

investigated the OS effect on kidney development after a kidney transplant. Thus, we have studied the OS effect on kidney function by determination of fatty acids metabolites generated by ROS action. In addition, after a thorough bibliographic review, we have not found an article which describes the monitoring of this specific type of metabolite in these patients.

In this regard, OS evaluation in patients receiving transplants has been carried out by monitoring four F₄-NeuroPs, two F₃-NeuroPs_{*n*-6 DPA}, and four F₂-dihomo-IsoPs at six different established times, which highlighted differences in their behaviors.

Focusing on the F₄-NeuroPs, metabolites produced from *n*-3 DHA by OS, all of them were detected only in patients receiving transplants. This shows that these metabolites were produced in higher concentrations than in the control group due to the increased inflammation and OS that this kind of patient exhibits, as observed by Lauzurica et al. [26]. 4(RS)-F_{4t}-NeuroP, 4-F_{4t}-NeuroP, and 10-F_{4t}-NeuroP did not follow a distinct pattern, showing increases and decreases that did not correspond to the kidney function; however, 10-epi-10-F_{4t}-NeuroP -the other NeuroP derived from *n*-3 DHA- showed a non-significant tendency to increase as time elapsed after the operation. We did not expect this tendency as the posttransplant phase progressed, which could reflect some specific pathophysiological mechanism of transplanted organ damage. Clinical appearances of chronic rejection are not noticeable until the existence of an advanced alteration of glomerular structure. Thus, the long-term urinary excretion of this F₄-NeuroP has to be studied in order to establish the relationship between its increase and the chronic rejection of the graft.

The F₃-NeuroPs_{*n*-6 DPA}, 4-epi-4-F_{3t}-NeuroP_{*n*-6 DPA} and 4-F_{3t}-NeuroP_{*n*-6 DPA}, produced from the breakdown of *n*-6 DPA by OS, didn't follow a common pattern. So, 4-F_{3t}-NeuroP_{*n*-6 DPA} increased

and decreased, with no correlation with the kidney function of patients, and showed no differences in concentration between the beginning and the end of the study. Moreover, this metabolite was the only one found in healthy patients, for some of whom it occurred at higher concentrations: this needs to be studied thoroughly, to have a better understanding of the metabolism of this biomarker.

On the other hand, 4-*epi*-4- $F_{3\tau}$ -NeuroP_{*n*-6} DPA was detected only in transplant patients, and its level clearly dropped as kidney function improved, with no significant differences in its concentration at different consecutive moments - whereas we found a significant decrease when comparing the worst and best moments regarding kidney function: 5 days and 6 months post transplant, respectively. In addition, significant and positive correlations were discovered for the 4-*epi*-4- $F_{3\tau}$ -NeuroP_{*n*-6} DPA concentration and Prot/Creat ratio at 1 month, 2 months and 6 months post transplant, as well as a slight relationship at 3 months post transplant. For 5 and 10 days post transplant, no correlation with urinary protein excretion was detected, probably because this parameter is not assessable in the first four weeks posttransplant. We assigned the decrease in the urinary level of 4-*epi*-4- $F_{3\tau}$ -NeuroP_{*n*-6} DPA, which depended on both the posttransplant period and the renal function improvement, to decreased OS in the kidney and to decreased proteinuria when the graft function after the ischemia period suffered at the time of the transplant become steady. The positive correlation between the Prot/Creat ratio and the 4-*epi*-4- $F_{3\tau}$ -NeuroP_{*n*-6} DPA concentration could be explained by increased oxygen consumption by nephron cells when protein charge increases due to absorption process [27,28]. Urinary albumin, the main globular protein in proteinuria, is reabsorbed by endocytosis that is mediated by the proximal tubular scavenger receptors megalin, cubilin, and CD36 [9-33] Subsequently, these receptor-albumin complexes activate protein kinase C signaling pathways, which lead to NAD(P)H oxidase-mediated ROS generation [34,35]. Thus, the lower the proteinuria the lower the concentration of ROS in proximal tubular cells; these species are among the most toxic cellular factors that directly induce tubule interstitial injury.

In the same sense, the reduction of urinary proteins excretion between the start and the end of the study might be due to the fact that during the transplant, and because of the ischemia-reperfusion phenomenon, in over 90% of transplant patients acute tubular necrosis and a variation of endothelial glycocalyx in the glomerular capillaries happened [36,37]. These variations involved an increase in proteins filtration that usually would be restored. The longer the ischemia period of the organ, the greater the glomerular damage by OS since the antioxidant defence mechanisms of kidney tissue, such as reduced glutathione and vitamin E, were weakened. Hence, the progressive restoration of the necrosis and endothelial damage suffered during transplantation decreased albumin filtration and subsequent reabsorption, with a reduction in ROS levels and therefore decreased damage by OS in proximal tubular cells.

Furthermore, F₂-dihomo-IsoPs, AdA derived metabolites, reflect the breakdown of this fatty acid by OS in the renal cortex and renal medulla [38,39]. This degradation reduces the concentration-dependent function of AdA on maintenance of vascular tone in renal and adrenal circulation, as Zhang et al. observed [39]. Its subsequent breakdown would cause incorrect vascularization of kidney tissue, disrupting its function and decreasing the life expectancy of the graft. We studied four metabolites which were detected in both transplant patients and healthy individuals. Three of them, 17-*epi*-17-F_{2 τ} -dihomo-IsoP, 17-F_{2 τ} -dihomo-IsoP, and *ent*-7(S)-7-F_{2 τ} -dihomo-IsoP were always detected at lower concentrations in transplant patients. This could be explained by the treatment. Kidney transplant patients in our institution are given high doses of corticosteroids. This therapeutic group has a wide range of effects and one of them is the phospholipase A2 inhibition. [40]. Unlike conventional prostanoids, these isomers are formed by OS injury in cell membranes and subsequently released after phospholipase activation [41]. Therefore, the inhibition of the enzyme

decreases the concentration of AdA metabolites.

The metabolites 17-*epi*-17-F_{2 τ} -dihomo-IsoP and 17-F_{2 τ} -dihomo-IsoP increased and decreased with no correlation with the allograft function. The other two metabolites of AdA studied, *ent*-7(RS)-7-F_{2 τ} -dihomo-IsoP and *ent*-7(S)-7-F_{2 τ} -dihomo-IsoP, decreased as kidney function improved and urinary proteins excretion decreased, with a minimum for both at 3 months post transplant - which coincided with the lowest proteinuria value. However, we only found a significant correlation between this metabolites and urinary proteins excretion at one of the six moments measured. The concentration of *ent*-7(S)-7-F_{2 τ} -dihomo-IsoP was always lower in transplant patients with respect to *ent*-7(RS)-7-F_{2 τ} -dihomo-IsoP. The *ent*-7(RS)-7-F_{2 τ} -dihomo-IsoP concentration was clearly higher in transplant patients at the beginning of the posttransplant phase, and at 6 months post transplant was similar for both groups. The decline in these two biomarkers shows how OS decreased as renal function improved. This finding could be used for monitoring of the kidney function. Moreover, these compounds should be studied in detail in acute rejection to see their possible predictive value in these events in which OS is increased [42].

On the other hand, our reference metabolite, 15-F_{2 τ} -IsoP, provided a similar behavior than the three metabolites of our study which could be considered as candidate indicators to monitor transplanted kidney function: 4-*epi*-4- $F_{3\tau}$ -neuroprostane_{*n*-6} DPA, *ent*-7(RS)-7-F_{2 τ} -dihomo-isoprostane, and *ent*-7(S)-7-F_{2 τ} -dihomo-isoprostane. We observed than 15-F_{2 τ} -IsoP decreased as kidney function improved and urinary proteins excretion decreased, and we discovered several positive correlations for the 15-F_{2 τ} -IsoP concentration and Prot/Creat ratio. These outcomes have been discussed before for the other three metabolites.

5. Conclusions

The analysis of F₄-NeuroPs, F₃-NeuroPs_{*n*-6} DPA, and F₂-dihomo-IsoPs in human urine provides a powerful approach to advance our knowledge of the role of oxidative stress in kidney transplant patients. Among the 11 biomarkers of lipoperoxidation measured, four of them, 4-*epi*-4- $F_{3\tau}$ -neuroprostane_{*n*-6} DPA, *ent*-7(RS)-7-F_{2 τ} -dihomo-isoprostane, *ent*-7(S)-7-F_{2 τ} -dihomo-isoprostane, and 15-F_{2 τ} -IsoP (8-iso-PGF_{2 α}) could be considered as candidate indicators to monitor transplanted kidney development in the short-term period after the surgical intervention. Unfortunately, little is known about these metabolites in this kind of patient and so more studies are needed to explain their influence on graft function and their possible ability to act as rejection predictors.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This work has been partially funded by the "Fundación Séneca de la Región de Murcia" Grupo de Excelencia 19900/GERM/15. We are grateful to Dr. David Walker (native English speaker) for your reviews of the English grammar and style of the current report.

References

- [1] N. Arulkumaran, N.M. Annear, M. Singer, Patients with end-stage renal disease admitted to the intensive care unit: systematic review, *Br. J. Anaesth.* 110 (2013) 13-20.
- [2] Lu Xin, David L. Perkins, A systems biology view of allograft rejection, *Curr. Opin. Organ. Transpl.* 10 (2005) 28-34.
- [3] M. Tonelli, N. Wiebe, G. Knoll, et al., Systematic review: kidney transplantation compared with dialysis in clinically relevant outcomes, *Am. J. Transplant.* 11 (2011) 2093-2109.

- [4] D.N. Cruz, H.R. de Geus, S.M. Bagshaw, Biomarker strategies to predict need for renal replacement therapy in acute kidney injury, *Semin. Dial.* 24 (2011) 124–131.
- [5] W.W. Williams, D. Taheri, N. Tolkoff-Rubin, R.B. Colvin, Clinical role of the renal transplant biopsy, *Nat. Rev. Nephrol.* 8 (2012) 110–121.
- [6] D.S. Wishart, Metabolomics: the principles and potential applications to transplantation, *Am. J. Transpl.* 5 (12) (2005) 2814–2820.
- [7] N. Schauer, A.R. Fernie, Plant metabolomics: towards biological function and mechanism, *Trends Plant. Sci.* 11 (2006) 508–516.
- [8] P.D. Whitfield, A.J. German, P.J. Noble, Metabolomics: an emerging postgenomic tool for nutrition, *Br. J. Nutr.* 92 (4) (2004) 549–555.
- [9] J.C. Lindon, E. Holmes, J.K. Nicholson, Metabolomics: systems biology in pharmaceutical research and development, *Curr. Opin. Mol. Ther.* 6 (3) (2004) 265–272.
- [10] J.T. Brindle, H. Antti, E. Holmes, et al., Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ¹H NMR-based metabolomics, *Nat. Med.* 8 (12) (2002) 1439–1444.
- [11] N. Serkova, T.F. Fuller, J. Klawitter, C.E. Freise, C.U. Niemann, H NMR-based metabolic signatures of mild and severe ischemia/reperfusion injury in rat kidney transplants, *Kidney Int.* 67 (3) (2005) 1142–1151.
- [12] J.R. Salaman, Monitoring of rejection in renal transplantation, *Immunol. Lett.* 29 (1–2) (1991) 139–142.
- [13] Saldaña Bernabeu Alberto, García Triana Bárbara Elena, Enamorado Casanova Antonio, García Piñeiro José Carlos, El estrés oxidativo en la fisiopatología del trasplante renal, *Rev. Cuba. Invest. Bioméd.* 18 (3) (1999) 225–230.
- [14] World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects, WMA, (2008), pp. 1–5 <http://www.wma.net/en/30publications/10policies/b3/>.
- [15] E.J. Lamb, C.R.V. Tomson, P.J. Roderick, Estimating kidney function in adults using formulae, *Ann. Clin. Biochem.* 42 (2005) 321–345.
- [16] S. Medina, I. Miguel-Elizaga, C. Oger, J. Galano, T. Durand, M. Martínez-Villanueva, M.L. Castillo, I. Villegas-Martínez, F. Ferreres, P. Martínez-Hernández, A. Gil-Izquierdo, Dihomo-isoprostanes-nonenzymatic metabolites of AdA-are higher in epileptic patients compared to healthy individuals by a new ultrahigh pressure liquid chromatography-triple quadrupole-tandem mass spectrometry method, *Free Radic. Bio Med.* 79 (2015) 154–163.
- [17] A.-L. Auvinet, B. Eignerova, A. Guy, M. Kotora, T. Durand, Total Synthesis of 4-F3t-Neuroprostane and its 4-epimer, *Tetrahedron Lett.* 50 (2009) 1498–1500.
- [18] C. Oger, V. Bultel-Poncé, A. Guy, L. Balas, J.-C. Rossi, T. Durand, J.-M. Galano, The handy use of Brown's catalyst for a skipped diyne deuteration: application to the synthesis of a d4-labeled-F4t-neuroprostane, *Chem. Eur. J.* 16 (2010) 13976–13980.
- [19] A. Guy, C. Oger, J. Hepekauzen, C. Signorini, T. Durand, C. De Felice, A. Fürstner, J.-M. Galano, Oxygenated metabolites of n-3 polyunsaturated fatty acid as potential oxidative stress biomarkers: total synthesis of 8-F_{3t}-IsoP, 10-F4t-NeuroP, and [D₄] – 10-F4t-NeuroP, *Chem. Eur. J.* 20 (2014) 6374–6380.
- [20] S. Medina, R. Domínguez-Perles, J.I. Gil, F. Ferreres, et al., A ultra-pressure liquid chromatography/triple quadrupole tandem mass spectrometry method for the analysis of 13 eicosanoids in human urine and quantitative 24 h values in health, *Rapid Commun. Mass Spectrom.* 26 (2016) 1249–1257.
- [21] C.V.F. Ortega, Avances en la calidad de vida relacionada con la salud y trasplante renal, *Nefrología* (2006) 157–162.
- [22] L.C. Corrales, M.M.M. Ariza, Estrés oxidativo: origen, evolución y consecuencias de la toxicidad del oxígeno, *Cienc. Bioméd.* 10 (2012) 213–225.
- [23] M. Coronado, S. Vega, R. Gutiérrez, B. García, G. Díaz, Los ácidos grasos omega-3 y omega-6: nutrición, bioquímica y salud, *REB* 25 (2006) 72–79.
- [24] M. Repetto, J. Semprine, A. Boveris, Lipid peroxidation: chemical mechanism, biological implications and analytical determination, *InTech* (2012) 3–30.
- [25] R. Castillo, P. Huerta, R. Carrasco, R. Rodrigo, Estrés oxidativo y daño renal, *CIMEL* 8 (1) (2003) 43–52.
- [26] R. Lauzurica, M.C. Pastor, B. Bayés, et al., F2-isoprostanes in kidney transplant patients: relationship with inflammatory markers, *Transplant. P.* 37 (2005) 3842–3843.
- [27] K.A. Nath, A.J. Croatt, T.H. Hostetter, Effect of dietary protein restriction on oxygen consumption and oxidant stress in the remnant nephron, *Kidney Int.* 33 (1998) 381.
- [28] I. Bruzzi, A. Benigni, G. Remuzzi, Role of increased glomerular protein traffic in the progression of renal failure, *Kidney Int.* 62 (1997) 29–31.
- [29] S. Tang, J.C. Leung, K. Abe, K.W. Chan, L.Y. Chan, T.M. Chan, K.N. Lai, Albumin stimulates interleukin-8 expression in proximal tubular epithelial cells *in vitro* and *in vivo*, *J. Clin. Invest.* 111 (2003) 515–527.
- [30] M. Morigi, D. Macconi, C. Zoja, R. Donadelli, S. Buelli, C. Zanchi, M. Ghilardi, G. Remuzzi, Protein overload-induced NF-kappaB activation in proximal tubular cells requires H(2)O(2) through a PKC-dependent pathway, *J. Am. Soc. Nephrol.* 13 (2002) 1179–1189.
- [31] M. Abbate, C. Zoja, G. Remuzzi, How does proteinuria cause progressive renal damage?, *J. Am. Soc. Nephrol.* 17 (2006) 2974–2984.
- [32] Y. Iwao, K. Nakajou, R. Nagai, K. Kitamura, M. Anraku, T. Maruyama, M. Otagiri, CD36 is one of important receptors promoting renal tubular injury by advanced oxidation protein products, *Am. J. Physiol.-Ren.* 295 (2008) 1871–1880.
- [33] M. Li, S. Balamuthusamy, E.E. Simon, V. Batuman, Silencing megalin and cubilin genes inhibits myeloma light chain endocytosis and ameliorates toxicity in human renal proximal tubule epithelial cells, *Am. J. Physiol.-Ren.* 295 (2008) 82–90.
- [34] M. Gekle, Renal tubule albumin transport, *Annu. Rev. Physiol.* 67 (2005) 573–594.
- [35] M. Arici, J. Brown, M. Williams, K.P. Harris, J. Walls, N.J. Brunskill, Fatty acids carried on albumin modulate proximal tubular cell fibronectin production: a role for protein kinase C, *Nephrol. Dial. Transplant.* 17 (2002) 1751–1757.
- [36] P. Martín, P. Errasti, Trasplante renal, *An. del Sist. Sanit. del Navar.* 29 (2006) 79–92.
- [37] S.H. Platts, J. Linden, B.R. Duling, Rapid modification of the glycocalyx caused by ischemia-reperfusion is inhibited by adenosine A2A receptor activation, *Am. J. Physiol.-Heart C* 284 (6) (2003) 2360–2367.
- [38] H. Sprecher, M. VanRollins, F. Sun, A. Wyche, P. Needleman, Dihomo-prostaglandins and -thromboxane. A prostaglandin family from adrenergic acid that may be preferentially synthesized in the kidney, *J. Biol. Chem.* 257 (7) (1982) 3912–3918.
- [39] D.X. Zhang, K.M. Gauthier, W.B. Campbell, Adrenergic acid metabolites as endogenous endothelium - and zona glomerulosa - derived hyperpolarizing factors, *Hypertension* 55 (2) (2010) 547.
- [40] C. Ji, C. Souvignet, N. Quirin, X. Grosbois, F. Bayle, Urinary F2-isoprostanes formation in kidney transplantation, *Clin. Transplant.* (2001) 58–62.
- [41] C. Guijas, A.M. Astudillo, L. Gil-de-Gómez, J.M. Rubio, M.A. Balboa, J. Balsinde, Phospholipid sources for adrenergic acid mobilization in RAW 264.7 macrophages. Comparison with arachidonic acid, *Mol. Cell. Biol. Lipids.* 1821 (11) (2012) 1386–1393.
- [42] Y.-Y. Mao, J.-Q. Bai, J.-H. Chen, Z.-F. Shou, Q. He, J.-Y. Wu, et al., A pilot study of GC/MS-based serum metabolic profiling of acute rejection in renal transplantation, *Transpl. Immunol.* 19 (1) (2008) 74–80.