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Biological variation and prognosis usefulness of new biomarkers in liver transplantation

Abstract

Background: An observational retrospective study has been conducted, including 52 patients (37 male and 15 female), ranging from 22 to 65 years old, who underwent an orthotopic liver transplantation (OLT) at the Hospital Universitario Central de Asturias (HUCA) between 2007 and 2010.

Methods: The main objective was to evaluate the post-OLT critical complication prognosis usefulness of the precursors of three new biomarkers: mid-regional proadrenomedullin (MR-proADM), carboxy-terminal-proendothelin-1 (CT-ProET-1) and mid-regional proatrial natriuretic peptide (MR-ProANP). As all of them are blood pressure mediators, stress-associated physiological phenomena are expected to affect their expression and secretion, mainly those related to blood circulation. Therefore, as a second goal, the biological variability of the biomarkers has been studied in a set of OLT patients without complications during the first postoperative week. The knowledge of the reference change value of the new biomarkers will be interesting for their correct interpretation in future investigations. The prognostic value of the new biomarkers was also compared to that of procalcitonin (PCT).

Results: It has been shown that the basal concentration of the biomarkers is higher in patients that undergo OLT than in the normal population, correlating with the severity of the pathology. The intra-individual biological variation of these biomarkers is similar to other biochemical parameters, the reference change value for OLT patients being 90% for CT-proET-1, 112% for MR-proADM and 127% for MR-proANP.

Conclusions: Multivariate analysis showed that MR-proADM was the best biomarker for the prognosis of severe complications.

Keywords: biological variation; liver transplantation; MR-proadrenomedullin.

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Introduction

Orthotopic liver transplantation (OLT) is a therapeutic procedure in which a failed liver is removed from the patient's body and a healthy donor liver is transplanted into the same location. OLT is indicated for end-stage liver diseases when other therapeutic approaches are not possible or have already failed and no absolute contraindications exist. The expected survival as well as the quality of life must also be lower than that potentially achieved after transplantation.

In Spain, the first liver transplantation was carried out in 1984 and, since then, our country has headed the list of transplant activity around the world in a number of interventions, reaching 25 liver transplants per million people [1].

The main complications of OLT are early hepatic dysfunction, infection, sepsis and rejection (Table 1) [2]. The way and degree in which such complications can modify the final success of a transplantation still remain unclear [3–5].

Acute rejections and infections are two important complications of OLT. Both situations usually appear within the first weeks after transplantation and their similar clinical findings make differential diagnosis difficult. The first sign of a potential rejection is usually a non-specific alteration of hepatic markers. Infection is the most frequent complication, being associated with a high post-transplant mortality rate [6].

Some recent references highlight the relationship between the expression of pro-inflammatory cytokines during transplantation and its clinical outcome. The plasmatic concentrations of IL-6 and TNF-α have been related to post-surgical complications [7, 8]. In fact, the systemic inflammatory response developed during the first days after the OLT event seems to be more potent when early

Immediate post-transplantation complications	Incidence, %
Techniques	26
Thrombosis/hepatic artery stenosis	4–12
Thrombosis of portal vein	1–13
Biliary complications	13–19
Reperfusion syndrome	20–30
Medical	75
Cardiac complications	5–30
Renal complications	30–50
Respiratory complications	60–70
Hepatopulmonary syndrome	25
Hepatorenal syndrome	20–30
Graft disfunction	5–10
Primary graft failure	
Infections	60–70
Acute rejection	25–30

Table 1 Incidence of the main immediate complications after OLT.

complications appear. The origin of post-transplant procalcitonin (PCT) induction remains unclear. The most widely accepted explanation is that both pro-inflammatory cytokines release and bacterial endotoxin contamination from the bowel flora during surgery are responsible for the post-OLT increase of PCT concentration [9–12]. This fact could also be related to ischemic-perfusion organ damage during surgery, as well as a partial hepatic origin of PCT, recently described not only as a marker but also as a mediator of infection [13].

The early identification of an acute rejection, and its differential diagnosis from infection, is still a clinical challenge of OLT. Several studies conducted with patients that underwent heart, pulmonary, renal or liver transplantation have indicated that PCT plasma concentrations increase during infection but not in rejection. This biochemical marker thus seems to play an important role in the differential diagnosis of both clinical processes [14, 15].

Two endothelium derived peptides, adrenomedullin (ADM), and endothelin (ET), with an antagonistic role in vascular tone regulation, have been described as crucial in the pathological pathway of Systemic Inflammatory Response Syndrome (SIRS). Other compounds with a very important role in blood pressure are the natriuretic peptides family, mainly the atrial (ANP) and B-type (BNP). All these new biomarkers have shown high concentrations in the presence of sepsis, opening promising expectations in prognostic and diagnosis of sepsis as a complement to the previous, already-known markers. The aim of the present work was to evaluate the post-OLT critical complication prognosis usefulness of the precursors of these three new biomarkers: midregional pro-adrenomedullin (MR-proADM), one of the most recently described markers with potential prognostic value in critically ill patients [16]; carboxy-terminal-proendothelin-1 (CT-proET-1) and midregional pro-atrial natriuretic peptide (MR-proANP), which are peptides described as markers for different postoperative cardiovascular and cardiac complications, respectively.

As all of them are blood pressure mediators, stress-associated physiological phenomena are expected to affect their expression and secretion, mainly those related to blood circulation (i.e., clamping and organ reperfusion). Consequently, as a second goal, the biological variability of the biomarkers was studied in a set of OLT patients without complications during the first postoperative week. Knowing the reference change value (RCV) of the new biomarkers will be of great interest for their correct interpretation in future investigations.

Materials and methods

An observational retrospective study was conducted, including 52 patients (37 male and 15 female), ranging from 22 to 65 years old, who underwent an OLT at the Hospital Universitario Central de Asturias (HUCA, Oviedo, Spain) between 2007 and 2010. The etiology of the cirrosis was alcoholic in 34% of the cases, viral (18.9%) and mixed (47.1%). An informed consent was obtained from each of the patients recruited, previously approved by the Ethical Committee at the HUCA.

Samples

Blood samples were drawn in sterile tubes containing EDTA-K3 as an anticoagulant. A minimum of four samples per patient were processed, drawn just before surgery, immediately after transplantation and every 24 h for the following 7 days. All the specimens were centrifuged at 2000 *g* for 10 min and plasma samples were then aliquoted and stored frozen at –80°C until analysis.

PCT, MR-proADM, CT-proET-1, and MR-proANP plasma concentrations were measured at the Clinical Biochemistry Laboratory (HUCA) in an automated Kryptor analyzer, using TRACE technology (Kryptor; BRAHMS, Hennigsdorf, Germany), without any pre-analytical treatments (i.e., extraction or derivatization). Analytical characteristics of the assays and reference values for the healthy adult population (Table 2) have already been described [17].

Reference change value was calculated according to the following formula [18]: $VRC=\sqrt{n}\times z\times\left(CV_A^{\,2}+CV_I^{\,2}\right)^{1/2}$, where z=1.64 is the unidirectional statistics for a 95% probability; CV_A is the coefficient of analytical variation; n is the number of samples and CV_I is the intra-individual coefficient of variation. In this calculus, the results observed in non-complicated patients during the first week post-transplantation (6 samples per patient) were included.

Non-parametric statistical tests were applied (Spearman's correlation, Kruskal-Wallis and Mann-Whitney U-tests) since the non-

	CT-proET-1	MR-proADM	MR-proANP
Stability			
At T≤−20°C, time	6 months	12 months	6 months
Freeze-thaw cycles, n	4	4	4
Valid samples	Serum		Serum
	Heparin	EDTA	Heparin
	EDTA		EDTA
Functional sensitivity	10 pmol/L	0.25 nmol/L	10 pmol/L
Measuring range	0.4–500 pmol/L	0.05–10 nmol/L	4.5–1000 pmol/L
Inter-assay CV	≤6.5%	≤11%	≤6.5%
Adult high reference limit	66.6 pmol/L	0.52 nmol/L	85.2 pmol/L

Table 2 Main analytical characteristics of the new biomarkers assays and reference cut-off values for the healthy adult population.

normality of the quantitative variables was confirmed (Kolmogorov-Smirnov test).

Bivariate and multivariate analysis were carried out to describe the potential relation between the new biomarkers and variables related to the basal disease severity (Child-Pugh scale and cirrosis etiology), the surgical complexity (cold, hot and total ischemic times, total admission time) or the concentration of classical biochemical tests (i.e., aminotransferases, bilirrubin, gamma glutamyl transferase, alkaline phosphatase and creatinine).

Receiver operator characteristic (ROC) curve analysis was applied to study the prognostic value of the new markers measured at 24 h post-OLT, compared to that of PCT. The ROC analysis were also performed taking into account the maximum concentration of the new biomarkers on the first postoperative week or a change over the basal value higher than the correspondent estimated RCV.

Statistical analysis was performed by SPSS v15.0. The significance level was stated at a p-value of 0.05.

Results

Six out of the 52 patients (11.5%) died during this period of time, whereas the remaining 46 (88.5%) survived. The surgery time ranged from 6 h 45 min to 22 h, 10 h being the mean intervention time. The mean cold ischemic time was 6 h (range 4–8 h), while the hot ischemic time lasted 1 h on average (minimum: 26 min; maximum: 1.5 h). Basal concentrations of all the three biomarkers were higher than the reference values described for the healthy population and correlated quite well with Child-Pugh's classification of severity ($p<0.01$), but not with the etiology of the cirrhosis.

According to the type of complication and diagnosis, the patients were classified into three groups:

1. Group I: patients without remarkable complications and good evolution (n=14);
2. Group II: patients with minor complications (n=14);
3. Group III: patients with major postoperative complications such as exitus (n=6), graft dysfunction or rejection (n=7), major vascular complications (n=9) and biliary complications (n=2).

With regard to the non-survivors, the causes of death were septic shock (n=1), primary liver failure (n=3; 2 of them were unsuccessfully retransplanted), hipovolemic shock (n=1) and biliary stenosis (n=1).

Although the classical tests of liver function did not show significant differences between the three groups of patients, significantly higher concentrations of PCT, total billirubin and creatinine were observed in the group of severe complications (group III). A similar trend was shown in the evolution of the new biomarkers during the days following OLT. Whereas CT-proET-1 did not reflect significant differences between the three groups, MR-proADM and MR-proANP showed significant higher values in patients from group III (Table 3). Since patients from groups I and II were considered an example of good post-OLT evolution, both the biological variation and the RCV of the new biomarkers were calculated based upon the results observed in this group of post-transplanted patients (Table 4). The range of the calculated RCV was 90%–127%.

Patients in group III showed very different patterns. For instance, Figure 1 shows the marker evolution in two patients who died during the first postoperative days. The first case (Figure 1, left) was a 53-year-old male patient who underwent OLT for mixed cirrhosis (ethylic and hepatocarcinoma). He suffered a primary graft failure and thrombosis 48 h after transplantation, so he was immediately retransplanted. Unfortunately, he died due to multi-organ failure and shock on the seventh day. A marked concentration increase for the three markers was observed on the second postoperative day, showing higher changes than the RCV (increase of 184%), but MR-proADM was already very high at 24 h post-OLT. The second example (Figure 1, right) was a 63-year-old female patient, also diagnosed with mixed cirrhosis (virus C and hepatocarcinoma). In the postoperative period, she suffered a hemoperitoneum with a massive bleeding and disseminated intravascular coagulation, needing surgery on the second post-OLT

	CT-proET-1, pmol/L	MR-proADM, nmol/L	MR-proANP, pmol/L	PCT, ng/mL	ALT, UI/L	AST, UI/L	GGT, UI/L	ALKP, UI/L	TBIL, mg/dL	CREA, mg/dL
Group I: Good evolution										
p25	60	0.92	99	0.6	303	219	42	52	1.05	0.51
Median	98	1.17	135	0.68	500	441	80	59	1.68	0.65
p75	108	1.61	219	1.55	913	807	127	97	2.8	0.83
Group II: Minor complications										
p25	82	1.11	130	0.58	295	310	47	58	2	0.5
Median	132	1.77	206	1.34	697	831	62	81	2.95	0.78
p75	184	2.43	355	4.1	1604	1880	135	100	4.29	1.23
Group III: Severe complications										
p25	120	2.01	232	1.54	451	454	52	60	1.68	0.76
Median	155	2.85	358	2.67	926	899	65	80	3.02	1.10
p75	224	4.50	728	6.93	2744	2956	173	147	8.77	1.67
p-Value	0.106	0.004[a]	0.006[a]	0.004	0.148	0.095	0.943	0.238	0.037[a]	0.005[a]

Table 3 Percentile distribution of the new biomarkers and several classic biochemical tests, observed in every group of post-OLT patients. [a]Non-significant differences between groups I and II. p25 and p75: 25th and 75th percentiles, respectively.

	CV_A, %	CV_I, %	RCV, %
CT-proET-1	4.6	19	90
MR-proADM	4.3	28	112
MR-proANP	3.1	31	127

Table 4 Biological variation data of the new biomarkers on good evolution post-OLT patients.

day. Afterwards, she showed primary graft failure due to ischemic problems, as well as important hemodynamic alterations and secondary sepsis. She died from multi-organ failure on the 13th day post-OLT. Both MR-proADM and MR-proANP showed significant changes on the second day with respect to the basal concentration, whereas the concentration of CT-proET-1 increased 24 h later.

Patients with rejection showed much lower increases of the markers, but some patients with severe vascular complications (Figure 2) showed changes similar to those of patients who died.

Bivariate analysis between the new biomarkers and other classic biochemical and clinical variables was carried out, a significant correlation being observed between the concentrations of the new biomarkers and renal dysfunction, as well as with the basal severity grade estimated using the Child-Pugh scale and the total admission time. With the multivariate analysis,

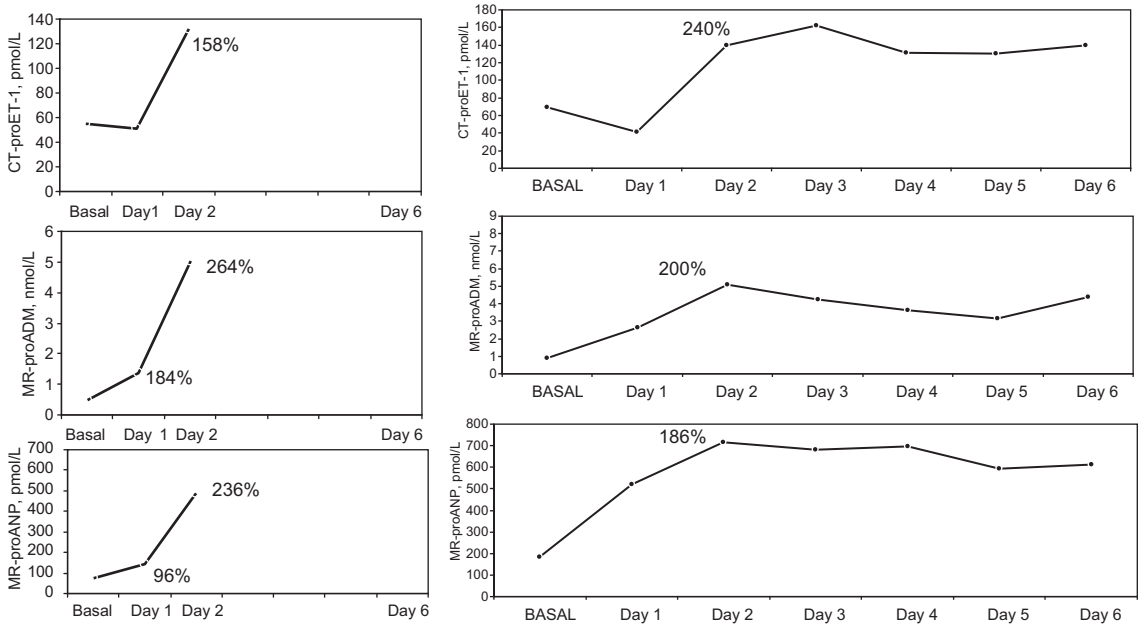


Figure 1 Evolution of the biomarkers in two patients who died during the first post-OLT time period.

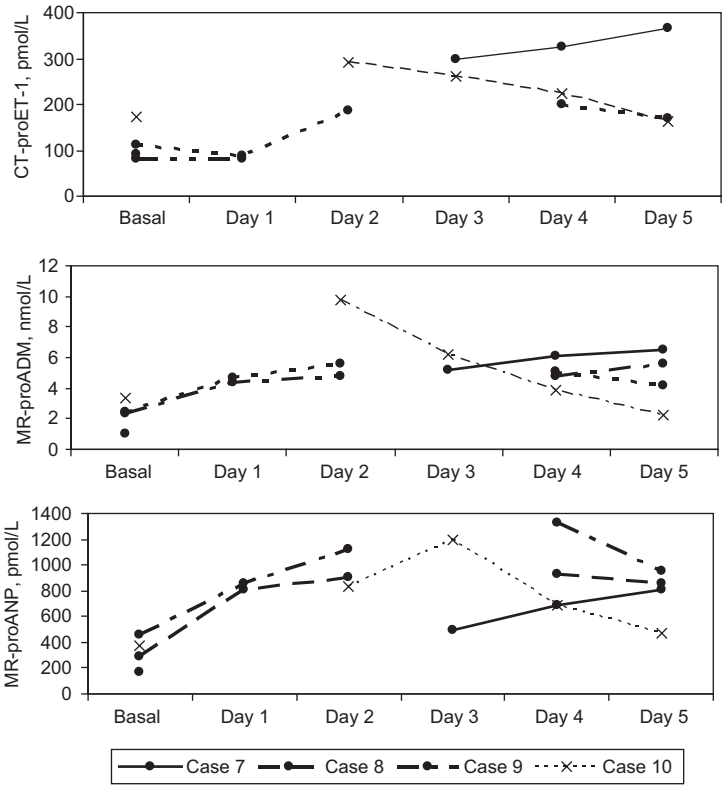


Figure 2 Evolution of the concentrations of the new markers in patients with severe vascular complications: stenosis of the cava vein (case 7), portal thrombosis (case 8), ischemic hepatitis (case 9) and intra-operative massive hemorrhage (case 10).

only creatinine concentration and total admission time remained significantly related to the marker concentrations ($p<0.05$).

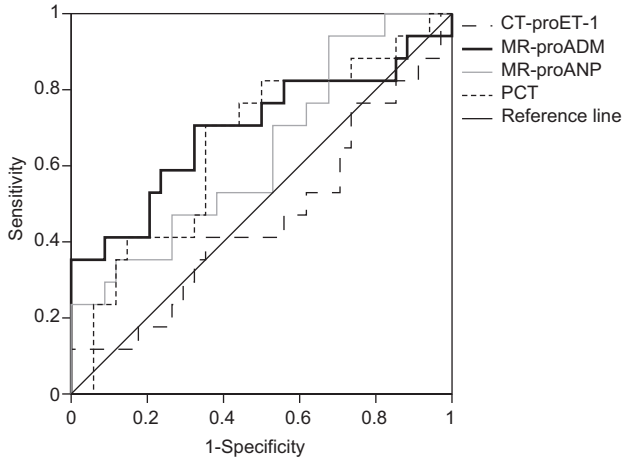
The potential prognostic value of the three biomarkers and PCT, measured 24 h post-OLT, was evaluated by ROC analysis for early severe complications on this OLT population (Figure 3). Only MR-proADM plasma concentration showed significant prognostic power, with an area under the curve (AUC) of 0.692 (95% CI 0.521–0.864).

However, taking into account the maximum concentration observed during the first week post-OLT, the prognostic value of MR-proADM increased to 77.9%. A sensitivity of 66% and specificity of 72% were obtained for a maximum MR-proADM concentration of 2.5 nmol/L as the best cut-off value. MR-proANP also showed ability to detect severe complications (AUC: 0.688), with similar performance for a cut-off concentration of 315 nmol/L (sensitivity: 72%; specificity: 60%). Similar prognostic power for severe complications, 0.767 (95% CI 0.61–0.923) was also found when it was observed an increase of MR-proADM over the basal value higher than the RCV. Moreover, such high elevations of the MR-proADM concentration have been shown to increase the risk of severe complications five-fold (95% CI 1.05–28.0) (χ^2, $p=0.03$). The same analysis showed no statistical signification with MR-proANP and CT-proET-1.

Discussion

Any surgical intervention produces an inflammatory response due to tissue damage. Consequently, close follow-up during the first postoperative days is essential to achieve differential diagnosis between the normal expected inflammation and other potential complications. Clinical evaluation is even more important in transplantation as an immediate intervention is needed in such complex patients and, moreover, there are antagonistic differences between the immunosuppressive treatment of a rejection and the potentiation of immunity needed in infection.

Classic biochemical tests of liver function (i.e., aminotransferase, bilirrubin, gamma glutamyl transferase and alkaline phosphatase) are usually ordered after transplantation, with postoperative normalization indicating a good prognosis, whereas prolonged serum increase concentrations on these tests reflect hepatic cellular damage. However, because marked concentration



	AUC	Standard error	p	95% Confidence interval	
				Lower limit	Upper limit
CT-proET-1	0.469	0.089	0.718	0.295	0.643
MR-proADM	0.692	0.087	0.026	0.521	0.864
MR-proANP	0.645	0.083	0.092	0.485	0.806
PCT	0.657	0.082	0.069	0.474	0.819

Figure 3 Prognostic value for early severe complications of PCT, CT-proET-1, MR-proADM and MR-proANP. Table shows the area under ROC curves (AUC) and their respective 95% confidence intervals.

changes of these conventional parameters are frequent during the immediate post-OLT period, correct interpretation is sometimes difficult. In this context, inflammation or infection markers, such as CRP and IL-6, can be interesting when an infection is clinically suspected, despite showing several limitations: low sensitivity and specificity and physiological increase in hepatic pathologies. In our experience, PCT is a useful marker not only for the differential diagnosis of sepsis but also for the prognosis of post-surgical OLT complications [19]. Nevertheless, a PCT elevation is also frequent after major surgery in the absence of infection, and a high cut-off is therefore needed. In a previous retrospective study including 118 OLT patients (data not shown), we found that a serum PCT concentration higher than 3.9 ng/mL, measured 24 h post-OLT, increased the risk of severe complications 16-fold (95% CI 5–54).

Some researchers have previously studied the evolution of ADM, ET and ANP in patients with hepatic pathology, after OLT [20–22]. Although altered kinetics and serum concentrations of these markers have been described in patients suffering postoperative complications, these previous reports used different methods (i.e., RIA, ELISA) and analyzed the entire hormone. One of the hypotheses of this study was that if the hormonal precursors (MR-proADM, CT-proET-1 and MR-proANP) showed at least similar usefulness, some advantages regarding their higher stability, total automatization and shorter time-around time could be obtained.

In fact, MR-proADM has recently been described as an interesting new marker for prognosis of severity, correlating with multi-pathology [16] and, similarly, both CT-proET-1 and MR-proANP are useful markers in different cardiovascular and cardiac complications, respectively [23, 24].

One of the main applications of the biological variation data is the estimation of RCV, defined as the minimum change that should be observed between two consecutive results of a marker to be considered as clinically relevant [25]. There are no previous references of biological variation for MR-proADM, CT-proET-1 and MR-proANP. However, since OLT constitutes very aggressive surgery, an RCV calculated for the healthy population might not be easily extrapolated. Therefore, it was considered interesting to ascertain the V_b data and the RCV of the new biomarkers in a post-OLT population without further postoperative complications (Table 4). Given that all three biomarkers play some role on blood pressure regulation and endothelial equilibrium, a high intra-individual variation according to factors such as hydration status and stress was expected. In fact, CV_I ranged from 19% to 31%, similar to levels described for other markers related to homeostasis (i.e., aldosterone, 29.4%; epinephrine, 48%) and not very different from some classic hepatic

biochemical tests, such as bilirrubin (23.8%) and ALT (18%) [26]. In the present study, the lowest RCV was found for CT-proET-1 (90%), followed by MR-proADM (112%) and MR-proANP (127%). With regard to other natriuretic peptides (BNP and NT-proBNP), previous studies described biological variations between 25% and 40% in patients with stable heart failure [27–29]. In the heart transplant population, our group found RCV of 97% for NT-proBNP [30] and 53% for MR-proANP (manuscript in preparation). Such a high RCV limits the actual usefulness of a marker, making it necessary to find very marked increases in order to obtain a prognostic value.

The retrospective evaluation of the concentrations of the studied biomarkers in severe clinical situations showed that the highest concentrations and changes with respect to the basal value were found in the group of non-survivors. Although in different populations, the relation between MR-proADM, MR-proANP and CT-proET-1 and mortality has already been described [31–33], with MR-proADM showing the highest discriminant power. As an example, Figure 1 shows the marker evolution of two patients who died during the first month post-OLT. The patient in Figure 2 on the left was the only one that suffered a severe immediate complication, requiring a retransplantation on day 2 due to thrombosis. He showed a high increase of the three biomarkers and very elevated concentrations of MR-proADM and MR-proANP. In the group of non-survivors (6 patients), an imbalance in physiological homeostasis markedly altered the biomarker concentration, perhaps related to processes of cell death and hypoxia.

The most frequent vascular complications after OLT include stenosis, thrombosis and ischemia. Severe ischemia or hemorrhage can lead to systemic hypotension with fatal consequences according to the severity of the lesion. Although an increase of vasoconstrictor molecules (MR-proADM) and a decrease of hypovolemic and hypotensor factors (CT-proET-1 and MR-proANP) could be expected, all three biomarker concentrations increased in those patients suffering vascular complications (Figure 2). It is possible that contrary effects are being added. Our results have shown that MR-proADM follows the same tendency as the functional hormone ADM [20] and, although the studied population is not large enough yet, the MR-proADM concentrations in severe vascular complications were significantly higher than in patients without complications.

To study the prognostic value of the markers, three groups of patients were established according to the complications that appeared during the first postoperative month. The concentration of each biomarker measured on the first postoperative control, as well as the maximum concentration observed during the first week, were studied to find a potential cut-off value for different clinical situations. Previous reports showed the role of MR-proADM as a marker of poor prognosis and death in heart failure patients and severe infectious complications [34]. However, the prognostic usefulness of natriuretic peptides for morbimortality has also been widely described in different clinical situations [35–39]. In fact, due to the essential role of neurohormonal activation in the physiopathology of heart failure, both MR-proANP and MR-proADM have been proposed as candidates with potential prognostic value.

In the present work, it was shown that in the first postoperative control, MR-proADM was the most efficient marker – even better than PCT – to predict severe complications. The lack of performance obtained here by PCT contrasts with that observed in an extension of a previous study [19] carried out only with PCT (88%) (data not published). However, the prevalence of severe cases in that cohort of patients was significantly lower than in the present work (i.e., 3% of exitus vs. 11.5%). This fact could explain the apparent discrepancy, the present results showing, furthermore, that MR-proADM is more robust to predict severity after OLT than PCT. Further studies comparing both biomarkers in a higher number of post-OLT patients are planned in order to verify this finding.

Finally, considering the maximum concentration observed during the first post-OLT week, the performance of MR-proADM increased to 78% and MR-proANP showed statistically significant discriminant power with an AUC of 0.688. The cut-off points selected according to the ROC analysis were 2.5 nmol/L for MR-proADM and 315 nmol/L for MR-proANP, similar to those previously described for other complications.

Conclusions

The concentration of the biomarkers in patients with chronic liver pathology (measured before OLT) is higher than the reference values and correlates with the severity of the disease.

The intra-individual biological variation of the new biomarkers is similar to that of other parameters with regulatory action. The RCV calculated for OLT patients was 90% for CT-proET-1, 112% for MR-proADM and 127% for MR-proANP.

Multivariate analysis of several clinical and analytical variables identified MR-proADM as the best of the new biomarkers for the prognosis of early and severe complications. However, the performance of PCT in the evaluation of post-OLT patients can be even higher than that of MR-proADM when less severe patients are followed-up.

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Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.

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