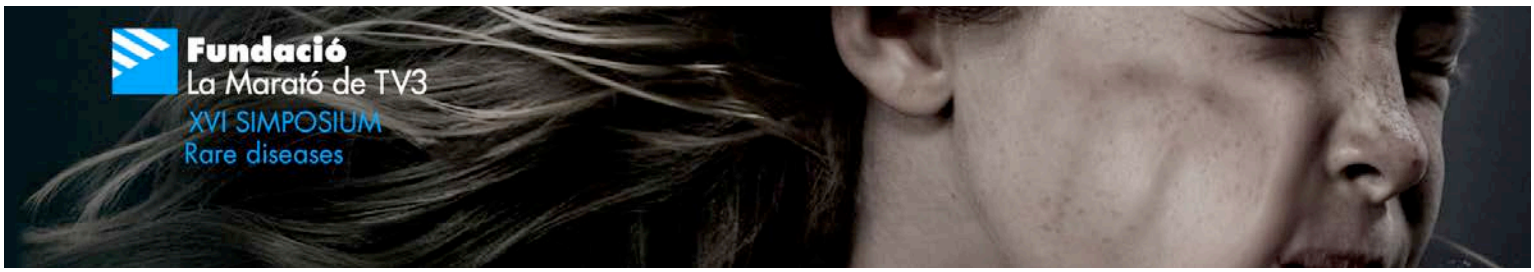

Proteomic analysis of post-translational modifications of transthyretin as an activity marker in patients with hereditary amyloidosis by TTR mutation: a case-control study

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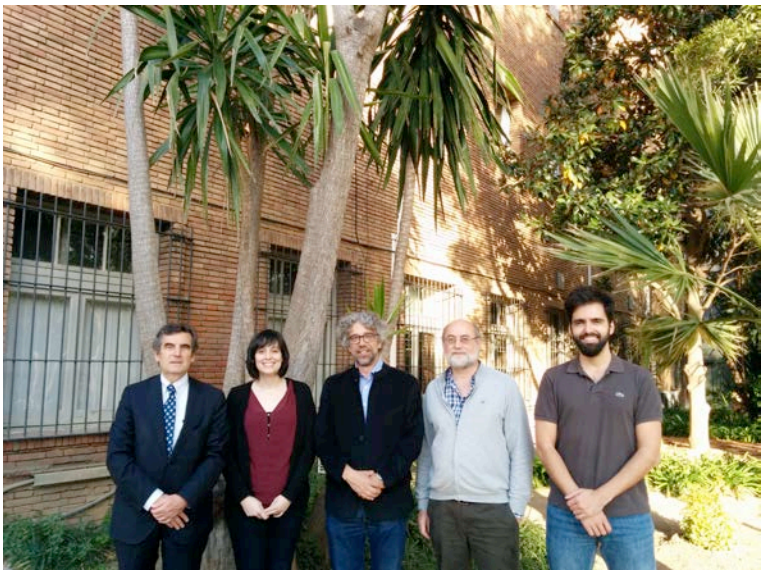

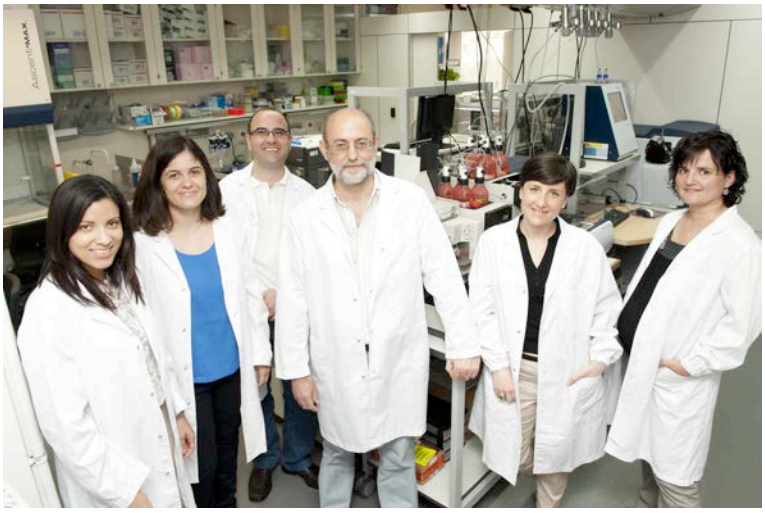

1. Summary of the project

There is ample evidence that the post-translational modifications (PTMs) of proteins are very important for their activity and processing. In this context and considering that recent discoveries of transthyretin (TTR) PTMs are relevant with respect to their amyloidogenic potential, we hypothesized that new metabolic changes in TTR can be identified and correlated with predisposition to develop the illness, the age of onset of symptoms, affected organs and severity of the disease. The overall objective of the project was to obtain new evidence about the molecular mechanisms associated with TTR that could be helpful on the one hand in the diagnosis and monitoring of disease and on the other in finding new therapeutic targets.

As preliminary objectives to determine a suggested correlation between post-translational modifications and amyloidogenesis in patients with pro-amyloidogenic variants of TTR, the first step was to define the study population. It initially consisted of a cohort of healthy controls (wild-type TTR producers) and 4 other patient populations defined by their carrier status of amyloidogenic TTR-V30M mutation or E89K, both asymptomatic and symptomatic (liver transplant recipients) and patients not receiving the liver of a patient afflicted with mutated TTR amyloidosis (domino liver recipients). Of these various cohorts, in addition to determining the status by genetic testing whenever required (healthy controls and potential carriers of TTR-V30M / E89K), a series of clinical (age of onset, affected organs, symptoms and severity) and paraclinical data (standard blood tests, electromyogram, electrocardiogram, echocardiogram) would be extracted.

At the same time, we would develop a methodology to define a method of preparing the serum sample that would recover all TTR PTMs by mass spectrometry techniques and subsequently evaluate the effect of these on the biophysical properties (stability, aggregation, amyloidogenicity) both of model peptides (synthesized de novo) from each mutated protein, and of wild-type and mutated whole TTR for the various mutations found (V30M, E89K, etc).

Simultaneously, plasma bank screening would be organized (Hospital Clínic) with samples from a cohort of known carriers of amyloidogenic TTR mutations, to later identify these PTMs

using the method that was developed. A subsequent statistical analysis would seek to correlate PTMs with known clinical factors from this hereditary TTR amyloidosis population and define its possible utility as a biomarker for diagnosing, monitoring and even guiding the therapeutic approach. Our work plan defined 4 tasks to complete, summarized as follows:

Task 1: To design a suitable cohort of individuals to study the influence of the TTR PTMs on different aspects of the disease (stage of disease: asymptomatic carrier, symptomatic patients before and after LT, age of onset of symptoms, number and type of organs affected, severity of organ involvement).

Task 2: To select the serum samples of TTR mutation-carriers from readily available samples in our blood bank and collect new samples from living and newly diagnosed individuals.

Task 3: Completion of clinical procedures of included individuals: clinical procedures as described in point 1.2.3 of the original protocol (briefly summarised as: clinical, prespecified blood analysis, electromyogram, electrocardiogram and cardiac ultrasound evaluations).
(4) Task 4: To correlate TTR PTMs with different clinical aspects of the disease.

Working plan modification

Given the difficulties of adding historical samples due to timing (> 3 years) and access to our biobank with a mandatory informed consent signed by the patients, and faced with an urgent need for quick access to a sufficient number of samples, we had to amend the original protocol with the inclusion of other hospital facilities including Hospital de Cruces, University Hospital Vall d'Hebron, Hospital de la Santa Creu i Sant Pau, University Hospital Bellvitge, Son Llàtzer Hospital in Majorca and University Hospital Puerta de Hierro in Madrid.

We also had to take into account the need to update the informed consent forms and the material transfer agreements (MTAs) required individually with approval from the various ethics committees. This was in addition to the complexity of accessing symptomatic and

asymptomatic carriers due to the rarity of this disease (it is not endemic in Barcelona, with most patients coming from outside the city) and the speed of clinical evolution in some patients, many of whom have marked gait difficulties especially in advanced stages. There were inherent difficulties in scheduling paraclinical exams from different departments, e.g. blood analysis, neurophysiology, echocardiography, electrocardiography.

As a result of all this, completion of tasks 1, 2 and 3, and the collection of all respective data, were delayed and finally slowed down, making it impossible for us to reach the target fully, achieving only 150 immunology bank samples, 32 ECGs, 36 EMGs and 37 cardiac ultrasound, even though we maximised coordination between our facility departments and with partner hospitals outside Barcelona. Likewise, the blood samples arrived sequentially and some late in the extension year, so the first bulk analysis of PTMs could be done only for the first 55 samples. However, after partially redesigning the protocol we managed to get 85% of the samples initially considered in just 12 months (Table 1).

In addition, the parallel therapeutic advances in ATTR changed the treatment paradigm in the last two years, from liver transplantation as the sole therapeutic possibility to the newly available TTR-stabilizers (tafamidis (on-label), diflunisal (off-label)) introduced between 2012-2013, and a few clinical trials for TTR-RNA silencing therapies. These alternatives changed the concept of cross-sectional analysis and forced us to reassess monitoring patients according to their clinical evolution with and without these treatments/clinical trial drugs (Tables 2 and 3), with the growing need to combine the stratification of symptomatic patients according to more precise staging systems such as Coutinho's (1980) and Yamamoto's (2007) (Table 1). Samples of 7 patients with mutation E89K could not be included in the initial analysis because more samples were needed to standardise this technique for this mutation (Article by M. Vila, related to this research).

2. Results

- We applied the two developed methodologies of proteomic analysis (targeted LC-MS and intact protein analysis) to the analysis of plasma samples from a cohort of patients with the TTR-V30M amyloidogenic mutation in asymptomatic and symptomatic carriers at different stages of disease progression (FAP), along with a control group of individuals, collected and selected as described in the original protocol (known healthy and non-TTR mutation carriers).

The main results derived from the analysis of the various TTR isoforms so detected are as follows:

- The levels of total TTR in plasma were significantly higher in all TTR-V30M carriers compared to controls. There were non-significant differences in these levels between symptomatic individuals at different progression stages of FAP. (Figure 5A from original work)
- The ratio between v30M-TTR and wtTTR is around 60:40 in all individuals carrying the mutation and this is independent of disease staging. (Figure 5B from original work)

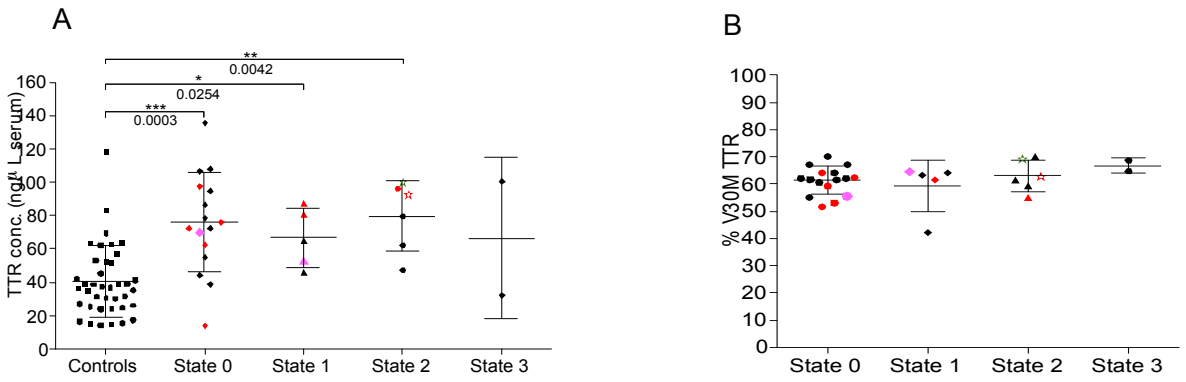

Figure 5. Targeted LC-MS analysis of TTR in plasma samples A) TTR total levels among controls and V30M-TTR carriers at different stages of FAP. B) Percentage of V30M-TTR in plasma among individuals at different stages of FAP.

- The plasma levels of the isoforms with free Cys-10 and those modified with S-Cys and S-sulfonation did not present significant variations during FAP progression, nor did they differ from controls.
- The plasma levels of S-CysGly and S-GSH TTR isoforms were significantly higher among asymptomatic V30M-TTR carriers (stage 0) than in those seemingly initiating progression (stages 1 and 2) (Figure 6 from original work). These levels could thus prove useful as markers of initial progression into ATTR. This result needs validation with a larger number of samples among these groups.

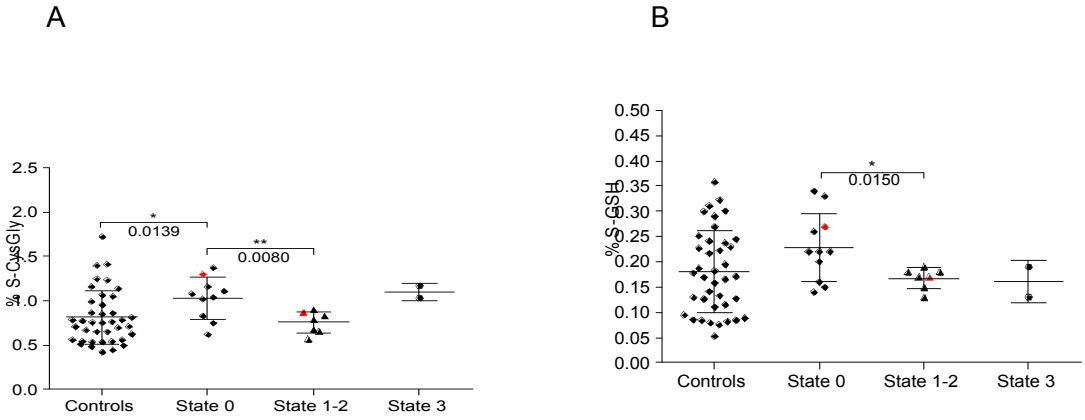

Figure 6. Targeted LC-MS analysis of Cys-10 PTMs in TTR plasma samples. A) Levels of S-CysGly isoforms among controls and V30M carriers at different FAP stages. B) Levels of S-GSH isoforms among controls and V30M carriers at different FAP stages.

- We applied these methods to the analysis of a cohort of samples from patients who had undergone liver transplantation and domino liver transplantation in cooperation with University of Lisbon and Hospital Curry Cabral of Lisbon.

From the analysis of a time series of samples taken at different times after liver transplantation we have been able to measure time-dependent replacement of V30M-TTR among V30M carriers who received a wtTTR producing liver and to determine the kinetics of appearance of V30M mutant protein in individuals with a domino-liver transplant from TTR-V30M producing donors (Figure 7A from original work). On the other hand, analysis performed long after the initial liver-transplant procedure allowed us to observe that levels of

total TTR among domino-liver transplant recipients is greater than in those FAP patients who received a wtTTR-producing liver (Figure 7B from original work). This result is in agreement with that observed in a group of samples described previously (data not shown).

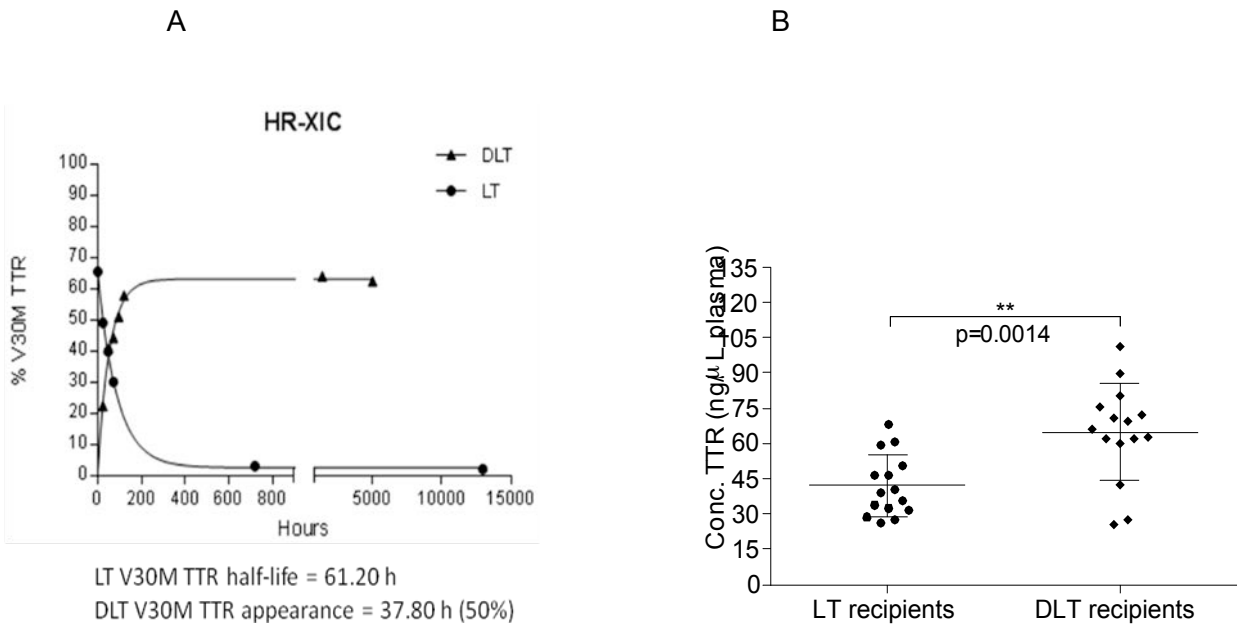

Figure 7. Targeted LC-MS analysis of plasma TTR. A) Levels of V30M TTR and wtTTR at different times after liver transplantation (LT) and domino-liver transplantation (DLT). B) Levels of total TTR in plasma in the long term after transplantation.

We have developed a methodology for quantification of PTMs in serum samples as well as for the determination of serum TTR levels from healthy (wt) and TTR-amyloidotic (V30M mutation) individuals. It involves an enrichment step by immunoprecipitation followed by mass spectrometry analysis of (i) the intact TTR protein and (ii) targeted LC-MS analysis of peptides carrying the PTMs of interest. Analysis of serum samples by the combination of the two methods affords complementary information on the relative and absolute amounts of the selected TTR PTM forms. It is shown that methods based on intact protein are biased for specific PTMs since they assume constant response factors, whereas the novel targeted LC-MS method provides absolute quantification of PTMs and total TTR variants.

The reported methodology has been applied to two different sets of clinical samples. Analysis of samples from FAP patients at different stages of the disease reveals changes in the distribution of the Cys-10 PTMs during FAP progression, some of those Cys-10 PTMs being possible biomarkers of disease onset. Through the analysis of a time series from FAP patients having undergone liver transplantation (LT) and from domino liver transplantation (DLT) recipients from V30M carriers, we have characterized the progression of the wt:V30M ratios, as well as the evolution of the Cys-10 PTMs, from transplantation and up to 9 years afterwards.

As a result of the human samples analysis, we preliminarily point out S-GSH and S-CysGly isoforms as biomarkers of disease progression. Particularly, we suggest that G-GSH and S-CysGly could be good indicators of the presence of soluble aggregates as a consequence of increased oxidative stress. We observed increased levels of the two Cys-10 PTMs in asymptomatic patients when compared to controls or in domino liver transplanted patients when compared to liver transplanted ones. More importantly, we propose that S-GSH and S-CysGly levels may be good indicators of FAP onset since the levels of the two isoforms are decreased after the appearance of the first FAP symptoms. The decrease in S-GSH and S-CysGly levels could be related to an increase in glutathione disulfide reductase (GSR) activity upon TTR deposition. Therefore, and despite being preliminary, the results presented are highly promising since they offer a non-invasive and objective measure of disease stage, which could enable an early intervention in FAP treatment.

3. Relevance and possible implications

Through the proteomic methods of analysis we have defined in this work, we have been able to analyse the plasma levels of the different modifications of the cysteine 10 residue in TTR. The analysis of TTR among asymptomatic and symptomatic carriers of the V30M-TTR mutation has revealed differential levels in of some of these modified forms.

If the obtained results are confirmed in a further study with a larger number of samples in this population, measuring the levels of these modified isoforms in plasma could be useful for determining early progress of ATTR in healthy carriers, and guide us to a prompt start of the currently available treatment options at the earliest stages of disease, thus increasing their efficacy.

4. Generated literature

Published articles

Marta Vilà-Rico; Núria Colomé-Calls; Luna Martín-Castel; Marina Gay; Sebastián Azorín; Marta Vilaseca; Antoni Planas; Francesc Canals. Analysis of post-translational modifications in human transthyretin associated with familial amyloidotic polyneuropathy by targeted LC-MS and intact protein MS**.** *Journal of Proteomics* (2015) in press.

International Communications:

M. Vilà-Rico, N. Colomé, G.Da Costa, C. Cordeiro, E. Barroso, A. Planas, S. Kaspar, P.O. Schmit, C. Baessmann, F. Canals
Analysis of post-translational modifications in human transthyretin associated with familial amyloid polyneuropathy by targeted LC/MS and intact protein MS
13th Human Proteome Organization World Congress (HUPO), Madrid, 5-8 October 2014

M. Vilà, N. Colomé-Calls, A. Planas, S. Kaspar, P. Olivier Schmit, C. Baessmann, F. Canals
Analysis of post-translational modifications in human transthyretin associated with familial amyloidotic polyneuropathy by targeted LC/MS and intact protein MS
EuPA 2013 European Proteomics Association, Saint Malo, France, 14-17 October 2013

M. Vilà, N. Colomé-Calls, A. Planas, S. Kaspar, P. Olivier Schmit, C. Baessmann, F. Canals

Analysis of post-translational modifications in human transthyretin associated with familial amyloidotic polyneuropathy by targeted LC/MS and intact protein MS

HUPO 12th Annual World Congress. The evolution of technology in proteomics. Yokohama, Japan, 14-18 September, 2013

M. Vilà, N. Colomé, F. Canals, A. Planas

Detection of post-translational modifications in human transthyretin associated with familial amyloidotic polyneuropathy by a SRM mass spectrometry method.

V Congreso de la Sociedad Española de Proteómica, Barcelona, February 2013.

Table 1. Basic demographic and clinical characteristics

Characteristics		**Overall (N=155)**	**Controls (N=70)**	**TTR-V30M (N=78)**			**Domino-LT recipients (N=7)**	**Pending Clustering (Other Hospital) (N=7)**
				Asymptomatic (n=29)	**Symptomatic (n=42)**			
					Non- LT recipients (n=25)	**LT-recipients (n=17)**		
Mean Age (SD), y			**45.8 (13.4)**	**43.6 (10.3)**	**63.6 (10.5)**	**45.6 (10.8)**	**70.3 (5.35)**	**55.1 (14.8)**
No (%)			**70 (45.1)**	**29 (37.2)**	**25 (32)**	**17 (21.8)**	**7 (9)**	**7 (9)**
Sex, N (%)	Male		41 (58.6)	12 (41.4)	16 (64)	8 (47)	4 (57)	NA
	Female		29 (41.4)	17 (58.6)	9 (36)	9 (53)	3 (43)	NA
	Sex ratio (M/F)		1.4	0.70	1.70	0.88	1.33	1.05
Disease Stage Median, [Coutinho]					**Stage 2**	**Stage 1**		
	1				12 (48)	12 (76.5)		
	2				8 (32)	2 (11.75)		
	3				5 (20)	2 (11.75)		
Disease Stage Median [PND]					**II**	**II**		
	I				12 (48)	8 (47)		
	II				4 (16)	4 (23.5)		
	IIIA				1 (4)	0 (0.0)		
	IIIB				4 (16)	1 (5.9)		
	IV				4 (16)	1 (5.9)		
CUS (% target)			**48 (56.47)**					

EMG (% target)			**49 (57.64)**					
ECG (% target)			**53 (62.35)**					
TTR-stabiliser use, No (%)					**5 (20)**			
	Tafamidis				4 (16)			
	male				3 (12)			
	female				1 (4)			
	Diflunisal				1 (4)			
	male				1 (4)			
	female				0 (0)			

Table 2. Longitudinal follow-up samples

Population Characteristics	Overall (N=113)	Male	Female
-Asymptomatic (n, %)	35 (33)	12 (34)	23 (66)
-Symptomatic (n, %)	45 (42.5)	36 (80)	9 (20)
-LT (n, %)	19 (17.9)	9 (47.4)	10 (52.6)
-Domino-LT (n, %)	7 (6.6)	4 (57)	3 (43)
-Other (pending definition – other facilities) (n, %)	7 (6.6)	NA	NA

Table 3. Sequential Sampling

Sequential Samples' characteristics	N (% of Total)
Overall Sampling	**42 (37)**
-Range	2-10
-Mean (sd)	3.2 (2.35)
Number of patients	**13 (11.50)**
-Asymptomatic	6
-Symptomatic:	6
NOT LT:	4
*Clinical Trial	2
*TTR stabiliser	2 (Diflunisal)
LT:	2
*Progression	1
*Stable	1

Abbreviations: NA, not available; USC, Cardiac ultrasound; ECG, Electrocardiogram; EMG, Electromyogram. LT, Liver-transplant.