A non-invasive method based on saliva to characterize TTR in FAP patients using FT-ICR high-resolution mass spectrometry

RAPID COMMUNICATION

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Abreviations:

AL - amyloidosis

ATTR - Transthyretin amyloidosis

FTICR-MS - Fourier transform ion-cyclotron resonance mass spectrometry

FAP - familial amyloid polyneuropathy

V30M - replacement of valine at position 30 by methionine in TTR sequence

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Abstract

Purpose: To identify, characterize and perform a relative quantification of human transthyretin (TTR) variants in human saliva. **Experimental design:** Serum and saliva samples were collected from healthy and familial amyloidotic polyneuropathy (FAP) patients, proteins separated by SDS-PAGE, TTR bands excised, in-gel digested and analyzed by MALDI-FTICR. **Results**: We identified and performed a relative quantification of mutated and native TTR forms in human saliva, based on FTICR-MS. The results are quantitatively identical to the ones obtained with human serum. In FAP patients subjected to cadaveric liver transplant, the TTR mutant form is no longer detected in saliva, while in patients receiving a domino liver from a FAP donor the mutant form of TTR becomes detectable in saliva, thus demonstrating the serum origin of TTR in saliva. **Conclusions:** Saliva TTR originates in serum and the ratio of mutant to native TTR is preserved. The method provides a non-invasive detection of mutated TTR and a relative quantification of TTR forms. **Clinical relevance:** Diagnostic and disease prognosis of FAP is crucial at early stages of the disease and after liver transplantation, the only curative therapy. A suitable non-invasive method was developed for monitoring the most important FAP biomarker in human saliva.

Statement of clinical relevance:

Familial amyloidotic polyneuropathy (FAP) is an autossomic dominant disease characterized by the formation of transthyretin (TTR) amyloid deposits. This crippling and fatal disease is associated to point mutations in TTR, the majority of cases being heterozygotic individuals. Detection of a specific mutation has been considered the most important parameter regarding disease diagnostic and prognosis, dictating the choice of therapeutic options. Serum TTR is mainly produced by the liver hence, liver transplant is the only curative therapy currently available. We established a non-invasive FTICR-MS based method to identify and characterize TTR and to perform a relative quantification of TTR forms in human saliva in a non-invasive way that provides an important monitoring parameter for FAP patients and for subjects enduring liver transplant, both cadaveric and domino.

Transthyretin amyloidosis (ATTR) is an autosomic dominant degenerative disease characterized by the formation of amyloid fibril deposits, mainly composed of transthyretin (TTR), in different organs and tissues [1,2]. These amyloid deposits hinder organ functions and ultimately lead to their failure. The disease is associated with single amino acid point mutations in TTR, a homotetrameric plasma protein responsible for the transport of thyroxine and retinol, the latter by the association with the retinol-binding protein [3]. The main hypothesis for ATTR pathogenesis is linked to the instability of the transthyretin tetramer favoring its dissociation to non-native monomeric species with the ability to self-associate. These soluble aggregates will evolve to insoluble aggregates and amyloid fibers with the characteristic β-cross sheet structure found in many neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [4]. There are over eighty known TTR point mutations, with a minority being non-amyloidogenic [5]. The most common amyloidogenic point mutation is the replacement of valine at position 30 by methionine, the V30M variant [6]. Carriers of this TTR variant are heterozygous in the large majority of cases and express both wild type TTR and TTR-V30M. Different point mutations result in distinct clinical prognosis and, since some therapeutic options are incompatible, the correct diagnosis at the earliest stages of the disease progress is crucial. As plasma TTR is produced in the liver, the only definitive therapy for ATTR is liver transplantation, therefore the detection and relative quantification of the two TTR variants in blood circulation, for heterozygous individuals, is essential to evaluate disease prognosis.

Several biological matrices can be used as a source of biomarkers to evaluate patient´s health such as blood, umbilical cord blood [7], meconium [8], amniotic fluid [9] and saliva [10]. In comparison to these matrices, saliva has the major advantage of being readily available and can be collected without invasive means. For patients, non-invasive collection techniques

dramatically reduce anxiety and discomfort and simplifies collection of repeated samples for monitoring over time. Saliva is also easier to handle for diagnostic procedures because it does not clot, thus lessening the manipulations required. Whole saliva contains proteins secreted by the salivary glands and proteins from the gingival crevicular fluid, a serum transudate present in the gingival crevice surrounding the teeth [11].

Previous studies have described the salivary proteome per se [12], while others described salivary dynamics or the differential abundance in saliva of single factors, such as TTR in the case of type II Diabetes [10]. In fact transthyretin concentration on saliva is a traditional marker of inflammatory and nutritional status. Murayama et al. (1999) evaluated and validated its suitability as a salivary marker of protein–energy malnutrition in elderly patients [13]. A reduced TTR amount in saliva has also been detected in patients suffering from head and neck squamous cellcarcinoma [14]. Urea stimulation (as a tannin) also leads to the increase of transthyretin and incidentally transferrin in saliva. Both proteins originate in saliva from serum [15].

A universal method, capable of identifying and relatively quantify TTR variants in circulation, using a totally non-invasive method with a significant higher cost/benefit to the patient would be highly valuable. Taking advantage of the ultra-high resolution provided by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS), peptide mass fingerprint coupled with FTICR-MS was used as a direct method for the identification and relative quantification of TTR variants in the human saliva.

Serum and saliva proteins from control and FAP patients with V30M mutation were analyzed by SDS-PAGE (Figure 1 A and B). In both cases, applying only ten micrograms of protein, which represents around 20 micro liters of saliva, is sufficient to distinguish the TTR protein band based only on Coommassie blue staining. Although no significant differences were observed

between serum SDS-PAGE protein profiles from control and FAP, we observed that the saliva protein profiles present some differences between these groups. These differences can be exploited to identify putative biomarkers for ATTR development, before the onset of symptoms. Concerning TTR levels, no differences were observed between control and FAP, as probed by Western blot analysis (Figure 1 C and D).

In contrast with serum, whole saliva is not constant in its composition, because of changes in salivary flow and a differential contribution from the different salivary glands. Thus, to evaluate TTR secretion along the day, saliva was collected from control individuals at different daily time points (Supplementary Figure 1). Although some variations were observed, TTR was always present in detectable amounts in all samples collected. The variability observed did not present a pattern common to all individuals (n=3), being characteristic for each individual.

Protein bands corresponding to TTR from serum and saliva of control individuals and FAP patients were excised and digested in gel with trypsin to perform peptide mass fingerprinting.

We were able to cover about 60% of TTR sequence (Supplementary Table 1). In both cases no differences were observed in TTR mass spectra from serum and saliva, indicating that the same TTR isoforms are present in both matrices (Figure 2a).

The 60% sequence coverage encompasses the amino acid sequences where the vast majority (more than 90%) of all known amyloidogenic TTR mutations occur, suggesting that an overwhelming majority of different amyloidogenic TTR variants can be unequivocally identified and quantitatively monitored by this methodology. In fact, we could detect two peptides containing the amino acid 30 which gives a higher confidence in the distinction between wild type and V30M TTR (Supplementary Table 1). In the control mass spectrum, a peptide with *m/z* 1366.759, corresponding to the sequence GSPAINVA**V**HVFR and a peptide with *m/z* 1494.853

corresponding to the sequence GSPAINVA**V**HVFRK, which represents a trypsin misscleavage, are clearly observed. The MALDI-FTICR mass spectrum of the TTR tryptic map obtained from the serum and saliva of FAP patients with a V30M substitution presents an additional peptide, not observed in the control mass spectrum at *m/z* 1398.732, corresponding to the 32.056 Da mass shift resulting from the V30M substitution (Figure 2). Hence, this peptide corresponds to the mutant peptide with the sequence GSPAINVA**M**HVFR, characteristic of the TTR-V30M variant. The presence of a methionine residue in this peptide sequence is further confirmed by the observation of a peak with a 17.002 Da mass increment from the mutant peptide (*m/z* 1414.726) (Supplementary Table 1), which corresponds to the oxidation of the methionine thioester. We could also observe a third peptide (*m/z* 1526.826*)* concerning the same sequence, but with a trypsin misscleavage (GSPAINVA**M**HVFRK).

The peptide characteristic of wild-type TTR (1366.759 sequence GSPAINVA**V**HVFR) is clearly observed in the sample of FAP individuals, as well as the peptide characteristic of V30M TTR variant. This shows, as expected for heterozygotic individuals, that FAP patients contain a mixture of wild type and V30M TTR variants in circulation. These results validate the strategy used to characterize TTR variants in human saliva, using a totally non-invasive methodology, opening the possibility to perform a relative quantification of both protein variants. The MS peak height of a single substance in comparison to that of the most similar internal standard can be used to quantify proteins, oligonucleotides [16, 17], peptides [18], oligosaccharides [19], and other low molecular weight compounds [20]. Some reports have been published, which show a linear response of the measured MS peak height ratio to the amount of analyte applied if a proteotypic internal standard is used [18, 21, 22, 23]. In this context, the relative intensity of peak with m/z of 1366.759 (wild-type TTR) to a TTR peptide present in all mass spectra

acquired (such as the peak of m/z of 1394.732, see Table 1) may be used to perform a relative quantification of both TTR variants in circulation. This comes from the fact that the peak 1394.732 corresponds to a peptide that does not contain any mutation so its intensity is a contribution from the two variants. The relative intensity of the peak 1366.759 to the peak 1394.732, is much lower in FAP individuals than in controls (Table 1), indicating that a lower amount of TTR WT is present in the serum of FAP patients. Thus, we can perform a relative quantification of these two variants in heterozygous individuals, since the overall quantity of TTR in serum is similar between the control and FAP individuals, as observed in a western blot analysis (Figure 1 C and D). This relative quantification is a significant advantage of this methodology over other methods, given the low amount of sample required.

By comparing the relative intensity of TTR WT and TTR V30M in serum and saliva, we could observe that they are very similar (ratio ≈1, Table 1). This observation validates the use of saliva to probe TTR levels in circulating TTR.

As plasma TTR is produced in the liver, the only definitive therapy for ATTR is liver transplantation. TTR form in circulation is readily substituted by the one of the transplanted liver. Sequential liver transplantation was introduced to recover the morphologically normal liver of a FAP patient as a donor to a patient suffering from liver fatal failure (as metastasis). The FAP patient receives a cadaveric liver and supplies his liver to the patient with metastasis. What is known so far is that TTR Met 30 was found in the serum of the recipient soon after the transplant. These patients are carefully followed since TTR Met 30 is in the serum and produced as it was in the FAP patient.

In this paper we also observed that after cadaveric liver transplantation and sequential liver transplantation TTR found in the saliva corresponds to the donor liver (Figure 2b).

The current development of diagnostic biomarkers (via proteomic and genomic approaches) in conjunction with technological developments in salivary diagnostics will lead to the development of robust diagnostic tools. To the best of our knowledge, this is the first time that TTR detection in human saliva is made both in control and FAP patients using high-resolution mass spectrometry (FTICR-MS). Especially, without the need for any previous enrichment of the proteins by immunoprecipitation, this method allows the detection in two days or less if using faster methods for protein digestion [24], of TTR variants using only 20 micro liters of saliva. This non-invasive methodology will allow the monitoring of TTR levels on FAP and patients after liver transplantation or sequential liver transplantation, with a higher cost/benefit to the patient. Since TTR WT and TTR V30M are both present in saliva at a ratio identical to the one found in serum, samples can be collected at any time of the day. With this method, we estimate that it will be possible to identify and perform a relative quantification in heterozygotic subjects in about 90% of all know TTR mutations in saliva. In the future, the identification of significant changes in saliva proteome composition associated with ATTR may represent potential diagnostic and/or prognostic markers.

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Figure legends:

Figure 1. SDS-PAGE separation of serum and saliva proteins and Western blot analysis of TTR from three control and three FAPFAP individuals.

2 – A – Peptide mass fingerprint of the TTR protein band after tryptic digest from serum and saliva. The ion **1366.759** is present both in control and FAP individuals. This corresponds to the peptide with the sequence GSPAINVAVHVFR. The ion **1398.732** is present only in FAP individuals. This ion corresponds to the peptide with the sequence GSPAINVAMHVFR **.** B - SDS-PAGE separation of saliva proteins and Western blot analysis of TTR from three patients transplanted with a liver from a cadaveric donor and three patients transplanted with a sequential liver. Peptide mass Fingerprint of the TTR protein band after tryptic digest from saliva from these patients. The ion **1366.759** is present both in control and FAP individuals. This corresponds to the peptide with the sequence GSPAINVAVHVFR. The ion **1398.732** is present only in FAP individuals. This ion corresponds to the peptide with the sequence GSPAINVAMHVFR

Table 1. Example of relative quantification of TTR in serum and saliva from FAP and Control individuals, using peak 1394.732 as internal standard.

	Mass	Control	FAP	Intensity	Ratio	Relative quantity	Ratio serum/Saliva
Serum	**1366.76**	√		$2{,}20 \times 10^6$	0.34	2.42	1.02
	1394.62	√		$6{,}40 \times 10^6$			
	1366.76		√	$0{,}80 \times 10^6$	0.14		
	1394.62		√	$5{,}85 \times 10^5$			
Saliva	**1366.76**	√		$1{,}60 \times 10^6$	0.43	2.38	
	1394.62	√		$3{,}70 \times 10^6$			
	1366.76		√	$0{,}80 \times 10^6$	0.18		
	1394.62		√	$5{,}85 \times 10^5$			

Figure 1

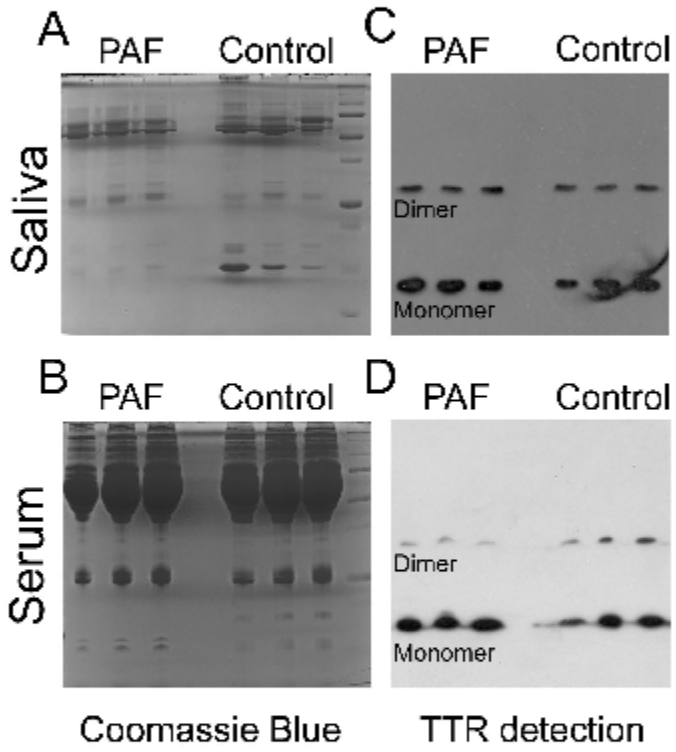
A
PAF
Control
C
PAF
Control
Saliva
Dimer
Monomer
B
PAF
Control
D
PAF
Control
Serum
Dimer
Monomer
Coomassie Blue
TTR detection

Figure 2

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