Rapid and sensitive determination of carnitine profiling by tandem mass spectrometry can be a diagnostic marker of paroxysmal atrial fibrillation

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Abstract

Aim: Paroxysmal atrial fibrillation (PAF), which is in the sub-group of atrial fibrillation that spontaneously resolves within 48 hours and does not last more than 7 days, is one of the most important causes of cryptogenic stroke. Other than ECG findings, there are no biochemical diagnostic criteria for PAF. Early diagnosis of PAF reduces the risk of morbidity and mortality. Therefore, it is important to identify new diagnostic markers. Metabolomics methods have recently started to be used for this purpose. Carnitine profiling with LC-MS/MS is a fast, inexpensive metabolomics screening method with high sensitivity and has started to be used for the determination of new diagnostic markers. The aim of this study was to investigate the viability of the carnitine profile as a new biochemical marker supporting diagnosis in PAF patients. Material and Method: A total of 27 carnitine ester profiles were examined with the LC-MS/MS method using serum samples taken from 41 patients diagnosed with PAF from ECG findings and 42 healthy individuals with sinus rhythm. Results: The C14, C16:1, and C18:1 serum carnitine ester levels of the PAF patients were determined to be statistically significantly higher than those of the healthy control group (p<0.05) and C6DC levels were found to be significantly lower (p<0.05). Discussion: The results of the study strengthened the view that with measurements made with LC-MS/MS, the carnitine profile, which is an inexpensive, sensitive reference method, can be used as an objective biochemical test to support diagnosis.

Keywords
Paroxysmal Atrial Fibrillation; LC-MS/MS; Carnitine Profiling; Rapid and Sensitive
Introduction

Atrial fibrillation (AF) is the most commonly seen type of arrhythmia [1,2]. Approximately 215,000 individuals are newly diagnosed each year in the European Union and it is predicted that there will be 14 million AF patients worldwide by 2030 [3-5]. When patients with complaints of palpitations at certain times present at clinics, the non-visualisation of findings on ECG increases morbidity and mortality [6]. Although there are several electrophysiological mechanisms that explain the onset and continuation of AF, the impairment of the cellular mechanism is not fully known [7,8]. Recent studies have reported that human persistent AF is upregulated by transcripts related to some glycolytic enzymes and when basic enzymes in energy metabolism and metabolism-related proteins are not corrected. Atrial fibrillation is known to originate from irregularities in energy metabolism at the cellular level [2]. Apart from ECG findings, there is no objective diagnostic criteria of PAF, which is a sub-group of AF that spontaneously recovers within 48 hours and does not last more than 7 days. PAF is one of the most important causes of cryptogenic stroke and is a difficult disease for clinicians to diagnose because of its insidious characteristics. Just as there are no objective diagnostic criteria that would suggest the presence of PAF, no biochemical marker specific to this disease has yet been identified [9,10].

Metabolomic studies aimed at obtaining new biochemical markers have recently become more important [11], due in part to the more widespread use of LS-MS/MS in routine laboratories. Metabolomics is the determination, identification, and quantification of metabolites using highly productive technologies. The cellular functions of metabolite levels are reflected in processed information. The aim of metabolomics is also to determine the metabolites that identify disease or enable monitoring of treatment [12-14].

L-Carnitine (3-hydroxy-4-N-trimethyl ammonio butanoate) is an amino-acid-like substance that has a function in the passage from the internal mitochondrial membrane of long-chain fatty acids that are physiologically synthesised in different tissues of a living organism and are to be transferred from the cytoplasm to the mitochondrial matrix [15]. Metabolomic studies aim to reveal the physiological processes of the body related to energy homeostasis and metabolism by identifying small molecules and metabolite levels. There has been insufficient research about metabolic profile disorders in PAF patients [16-18]. The identification of impaired carnitine metabolism provides a clue to reversing the metabolic effects in the circulation of the disorder, thus providing a new dimension for treatment. The focus of this study was to investigate whether identification of the carnitine profile with the rapid, inexpensive, reliable LC-MS/MS method from serum samples of PAF patients could be used as a new biochemical marker for this disease, which is asymptomatic and may result from a metabolic defect.

Material and Method

Study participants and sample collection

From a total of 120 patients who presented at the Cardiology Polyclinic with complaints of palpitations, 120 patients were excluded from the study because of sinus tachycardia, 18 with supraventricular tachycardia, 17 with ventricular tachycardia, 8 with ventricular extra-systole, 1 with multifocal atrial tachycardia, and 17 with other exclusion criteria. Thus, the study included 41 patients with a mean age of 66.9 ± 11.49 years diagnosed with PAF according to the European Society of Cardiology (ESC) 2016 guidelines and 42 healthy volunteers with a mean age of 65.60 ± 13.38 years with normal sinus rhythm. Patients or control subjects were excluded if they were aged <18 years, were pregnant, had liver disease, infection, active malignancy, hypertension, diabetes mellitus, or a history of stroke. The demographic data of the participants and the ECG findings are presented in Table 1.

| Table 1. Characteristics of the PAF group and the control group |
|-------------|-------------|-------------|-------------|-------------|
| Group       | N       | Mean±SD     | p-value |
| AGE         | Patient 41 | 66,90±11,49 | 0,189     |
|             | Control 42 | 65,60±13,38 |           |
| SIZE        | Patient 41 | 166,95±9,92 | 0,120     |
|             | Control 42 | 170,44±10,67|           |
| WEIGHT      | Patient 41 | 75,91±12,30 | 0,231     |
|             | Control 42 | 72,85±10,40 |           |
| BMI         | Patient 41 | 28,28±3,86 | 0,078     |
|             | Control 42 | 25,16±4,14 |           |
| PULSE       | Patient 41 | 65,55±8,63 | 0,056     |
|             | Control 42 | 78,62±5,71 |           |
| EF          | Patient 41 | 55,90±4,33 | 0,067     |
|             | Control 42 | 57,88±4,61 |           |

The study was conducted in accordance with the principles of the Helsinki Declaration. Informed consent was obtained from all the participants. Blood samples were obtained in the morning after 8 hours of fasting, withdrawn into gel tubes, and centrifuged at 500 rpm for 10 mins. The separated sera were placed in Eppendorf tubes and stored at -80°C until assay.

Chemicals and reagents

The reagents used were the internal standard set of Labeled Carnitine Standards-Set B, from Cambridge Isotope Laboratories (UK). Mobile phase modifiers of formic acid and Acetonitril were used, along with HPLC gradient grade methanol (J.T. Baker, Center Valley, PA, USA) and deionized water (Millipore Simplicity UV water purification system, Waters Corporation, Milford, MA, USA).

LC-MS/MS determination of carnitines

From the serum samples, 27 parameters of carnitine esters were measured with a Shimadzu Nexera X2 ultra high performance liquid chromatograph (UHPLC) coupled with a Shimadzu 8040 triple quadrupole mass spectrometer (MS/MS) (Shimadzu North America, Columbia, MD, USA). The carnitine profile was examined by modifying the newborn screening method developed by LaMarca and Azzari [19,20]. Filter paper (Whatman filter paper 10538018) cut into 3.2mm discs was placed in 96-well plates. Then a 5µl serum sample was placed in each well and left to dry overnight at room temperature. Sample extraction was achieved using 300 µL extraction solution of a mixture of methanol and aqueous solution of 3 mmol/L hydrazine hydrate hydrazine at an approximate relative volume/volume
ratio of 66.6% and 33.3%, respectively. The extract solution contained internal standards and stable heavy isotope analogues of several carnitines and acylcarnitines. The extracted sample was injected into Shimadzu LCMS-8040. Using a neutral loss scan of 46 Da in positive mode (CE-15V), mass spectral data for the amino acids were obtained. For the acylcarnitines, mass spectral data were obtained using a precursor ion scan of 85 m/z in positive mode (CE-25V). In each analyte, the percentage recovered was determined by comparison with an internal standard for each analyte. The Standard Concentrations were in the range of 7.6-152 µmol/L for acylcarnitines. Spiked samples with different concentrations of analyte were used as a daily control quality test.

Analysis Condition
Run of 2.2 minutes in FIA Flow 0.070 µL/min (A: Water+0.05% of Formic Acid, B: Acetonitrile, A/B: 30%/70%). 40 µL of sample injected Column Oven 30°C, Desolvatation Line 300°C, Heat 500°C, Nebulizing Gas 3 L/min, Drying Gas 20 L/min. All data collected were reprocessed using Shimadzu Neonatal Software, which automatically calculated the concentration of each compound.

Statistical Analysis
Analyses of the data obtained in the study were made using SPSS 20 for Windows software (SPSS Inc., NY, USA). To test for equality of variance, the data were statistically analyzed with the Levene test and the Shapiro Wilk test was used to evaluate the normality assumption. To determine differences between the groups, the Student’s t-test and Mann-Whitney U-test were applied. A value of p<0.05 was accepted as statistically significant.

Results
Serum carnitine profiles
Free carnitine and carnitine ester profiles were obtained from the serum samples of the PAF patients (n=41) and the healthy control group (n=42). No significant difference was determined between the groups in respect of body mass index values (Table 1).

After determining the concentrations of 27 carnitines measured in the serum samples, statistical analyses were applied. The carnitine concentrations of the serum samples analysed are shown in Table 2. The results obtained in the carnitine profiling were statistically analysed with a single variable to compare the endogenous components in the serum of the PAF patients with the profiles of the control group. The single variable statistical analyses showed which variables (carnitines) had different levels in the samples obtained from the PAF patients compared to the control group. Of the 27 carnitines examined in the serum samples, statistically significant differences were determined in C6DC, C14, C16:1, and C18:1 (p<0.05). Of these, significantly lower levels of C6DC and significantly higher C14, C16:1, and C18:14 levels were determined in the PAF group (Figure 1).

Discussion
Atrial fibrillation (AF) is a common cardiac arrhythmia seen together with increased risk of stroke, heart failure, myocardial infarct, and dementia [21]. Paroxysmal atrial fibrillation (PAF) is an AF sub-type with an insidious course that is difficult to di-
Carnitine profiling and PAF

Carnitine (3-hydroxy-4-N-trimethylaminoobutyric acid) is an important molecule with a function in the transport of long-chain fatty acids from the cell cytoplasm to the mitochondrial matrix. Carnitine deficiency is related to the accumulation of superfluous acyl-CoA esters and impairment of the intermediary metabolism [27,28]. Carnitine quantification is important as a complementary test for the diagnosis of uncommon metabolic diseases, including fatty acid destruction disorders [29]. In addition, several experimental models have shown that the damage associated with ischaemia or reperfusion of the myocardium is reduced with carnitine use [27,30]. Mondillo et al. showed that carnitine increased the potential of anti-arrhythmic drugs in the treatment of ventricular extrasystole [31]. In a study by Martina et al., the use of L-carnitine was determined to have an anti-arrhythmic effect in arrhythmias formed in acute myocardial infarctus [32]. In a study of diabetic mouse hearts, Broderick et al. reported that carnitine and derivatives corrected heart functions and increased tolerance to ischaemia [33]. In research similar to the current study, using the measurement method from dried blood, Zhansheng et al. partially showed that carnitine and esters could be used in the differentiation of haemorrhagic and ischaemic stroke [34]. In another study, Jeganathan et al. reported that mitochondrial dysfunction could play a role in postoperative atrial fibrillation [35].

A certain level of carnitine in the blood has a positive effect on cardiac contractility [31]. In the current study, measurement of 27 carnitine esters in the serum showed a significantly lower level of C6DC and significantly higher levels of C14, C16:1, and C18:1 in the PAF patients compared to the control group. Within these four parameters, the increase in the serum level of the carnitines esterised with long-chain fatty acids (C14, C16:1, C18:1) was noticeable (Figure 1). This significant difference could be related to arrhythmia caused by metabolic activity of new signal foci of carnitines that function as carriers in fatty acid transfer to the mitochondria in the indirect provision of energy. The increased electrophysiological activity in the new signal foci could be related to changes in carnitine metabolism. The LC-MS/MS mass spectrometry method is a reference method used in the measurement of carnitine levels.

As this method can measure tens of parameters with a single injection, it is accepted as more rapid and cost-effective than colorimetric and immunoassay methods. In the current study, more robust data were obtained about the metabolomics of carnitine with the LC-MS/MS device as a rapid, reliable, inexpensive reference method from the dry blood samples of this disease group. In the pathogenesis of AF, although the role played by various biochemical molecules has not been fully clarified, that the carnitine esters were found to be higher in the PAF patients of this study compared to the control group suggests that there is a carnitine metabolism disorder at the mitochondrial level. In the carnitine profile scans of this study, the increase in the C16:1 and C18:1 serum levels from the carnitines esterised with long-chain fatty acids is seen in Carnitine palmitoyltransferase II (CPTII) enzyme defect of metabolic diseases and the increase in C14 is seen in Very Long-Chain Acyl-CoA Dehydrogenase deficiency [36,37]. Although researchers have attempted to explain the emergence of different new electrical foci from the sino-atrial node with electrophysiological theories, no theory has been established that these types of arrhythmia could emerge in carnitine metabolism disorder. Despite there being no primary association between PAF etiology and carnitine ester levels, those levels change in the presence of other metabolic diseases and thus they are used by metabolism specialists for diagnostic purposes [38,39]. The data obtained in this study strengthen the view that carnitine profile screening can be used in patients with suspected PAF.

Conclusion

In this study, LC-MS/MS-based metabolomics was applied successfully to reveal the differences in metabolites between PAF patients and the healthy control group. Carnitine esters found at a significantly high level in dry blood samples can be used as a biomarker to assist in the diagnosis of PAF with severe complications, which is progressing asymptomatically and for which there is no diagnostic method other than ECG. By revealing that the pathogenesis of PAF could be clarified with the identification of carnitine metabolic pathways and new treatment protocols could be formed with intervention in these pathways, this study can be considered to be of value and guidance for further studies.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.
Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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