Is Alanine a biomarker for differentiating single vessel, double vessel and triple vessel coronary artery disease? - An in-vitro proton MR study.

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Objective: To identify biochemical markers to distinguish between controls and different forms of coronary artery disease (CAD), namely, single vessel disease (SVD), double vessel (DVD) and the triple vessel disease (TVD) using in-vitro MRS.

Introduction: Coronary artery disease (CAD) is a major cause of mortality and morbidity worldwide [1]. In general, an increase in the levels of plasma VLDL (very low density lipoprotein) and LDL (low density lipoprotein) and decrease in the HDL (high density lipoprotein) levels has been found to be associated with the development of CAD [2]. High levels of cholesterol and low density lipoproteins (LDL) in the blood leads to buildup of a fatty substance called plaque on the inner walls or lining of the arteries because of which coronary arteries become hardened and narrowed. The disease is classified in three categories based on the blockage in the number of vessels such as single vessel disease (SVD), double vessel (DVD) and triple vessel (TVD). The severity of disease increases with the increasing number of blockages. The most reliable test for diagnosing CAD is coronary angiography. Recently, methods exploiting biochemical changes associated with the development of CAD have been explored in order to develop a non-invasive robust method for unambiguous diagnosis of different forms of the CAD [3]. Pattern recognition approach and principal component analysis have been applied to study CAD using NMR [3]. The present study aims to determine the biochemical markers in whole blood plasma and the perchloric acid and acetonitrile extracts of the blood plasma to differentiate among various forms of CAD and controls using NMR spectroscopy.

Patients, Sample collection and preparation: Five ml of venous blood was drawn from subjects (n=112) undergoing angiography at the Department of Cardiology. The categorization of the patients as SVD, DVD and TVD was carried out based on angiography. The investigators, who carried out NMR spectroscopy (AM and US) were blind to the diagnosis of disease. The angiographically normal patients served as controls (mean age, 52 ± 11.9 years). The mean age group of the patients recruited was (56.1 ± 11.4 years). Forty-three samples [angiography normal controls (n = 9), SVD (n = 7), DVD (n = 12) and TVD (n = 15)] were investigated using perchloric acid extraction. Eighteen samples [SVD (n = 5), DVD (n = 4) TVD, (n = 5) and controls (n = 5)] were studied using acetonitrile extraction. The proton NMR experiments were carried out at 400 MHz [(DRX-400 (BRUKER, Switzerland)]. Typical parameters for one-dimensional experiments were: data points = 32 K, spectral width = 4000 Hz, number of scans = 32, relaxation delay = 14 s. Spin echo spectra were acquired with the echo times of τ = 16; 32 and 64 ms. The typical parameters used for DQF-COSY were: 2 K data points, spectral width 4000 Hz and 512 time domain points collected in t_1 dimension using 64 acquisition and relaxation delay of 2.5 seconds. The concentration of metabolites were determined by comparing the integrated intensity of isolated resonance of the metabolite of interest with that of TSP. 6 mM sodium formate added externally to the sample was used as a reference in CPMG experiments. Statistical analysis was carried out using ANOVA along with Bonferroni corrections. The institute ethics committee approved the study.

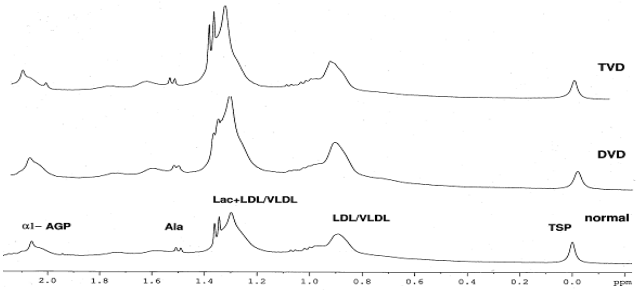

Table: Normalized intensities of metabolites in controls, and patients with CAD

Metabolites	Controls (n=13)	SVD (n=20)	DVD (n=19)	TVD (n=21)
LDL+VLDL	31.7±6.0*	32.0±3.7*	37.7±7.3*	39.2±8.7*
Ile/Leu/Val	18.9±4.0*	19.4±3.5	22.2±4.9*	24.2±3.4*
Lac	3.9±0.8	3.9±1.0	4.5±1.2	4.4±1.1
Ala	5.6±1.0	6.7±1.9*#	8.4±2.7*#	9.5±3.5*#
α-1 AGP	14.2±3.0*	16.7±3.8	18.0±6.0*	18.4±6.0*
α-Glc	3.8±1.4	4.4±1.2	5.0±1.6*	4.8±1.8*

* p- <0.05, controls vs.CAD; # p- <0.05 among SVD, DVD and TVD

Results Figure above shows the representative 1D proton NMR spectra of whole blood plasma sample from a control and patients with DVD and TVDCAD. In all, eighteen metabolites could be unambiguously identified. The intensities of various metabolites were normalized to the intensity of TSP for angiography normal and CAD patients are shown in Table. Elevated levels of several metabolites such as LDL/VLDL, Ile/Leu /Val, Ala, α-1AGP and Glc were in CAD patients compared to controls. The LDL/VLDL levels were significantly lower in SVD compared to DVD and TVD ($p<0.05$). Absolute concentrations of metabolites obtained through perchloric acid and acetonitrile extraction procedures showed that the concentration of Ala, Ile/Leu/Val and Glc were significantly higher in patients with TVD compared to controls. The levels of alanine (Ala) were significantly different between the three forms of CAD (see Table).

Discussion Our study demonstrated an increase in the intensity of LDL/VLDL in patients with CAD in comparison to angiography normal subjects. The intensity of LDL/VLDL was found to increase with the severity of the disease. There is strong evidence that LDL/VLDL is important determinant of the risk of CHD [4]. The most interesting finding of the present study is the statistically significant increase in the concentration of Ala in the CAD patients compared to angiography normal subjects. In CAD patients, Ala release may be related to the severity of coronary artery stenosis [5]. Carlsen et al reported that there is no net uptake and release of amino acids by the human heart except Glu and Ala. [6]. There are several ways in which amino acid exchange may be beneficial to the ischemic cell. Ala may act as a non-toxic carrier of ammonia that accumulates rapidly during anoxia. Shunting of pyruvate to Ala instead of Lac may diminish Lac accumulation and thereby relieve inhibition of glycolysis during ischemia. Amino acids may act fuel both during aerobic and anaerobic conditions but energy production from amino acid fermentation remains negligible compared with that from glycolysis. The most important advantage is the transport of reducing equivalents across the mitochondrial membrane to reoxidise cytosolic NADH via the malate-aspartate cycle. It has been reported that in a normal myocardium Ala is formed from Glu only during pacing stress but not at rest, whereas, the diseased heart has been shown to release Ala in both conditions during pacing stress and at rest [7]. Our study showed a significant increase in Ala concentration in CAD patients. These observations suggest the hypothesis that the chronic or repetitive bouts of myocardial ischemia may induce alterations in myocardial amino acid metabolism. Hence measurement of myocardial exchange of Glutamate; Ala and Lac can be suggested as a sensitive biochemical test for assessing myocardial ischemia, though more studies needs to be carried out in a larger cohort of patients.

References: [1] National Statistics Series DH1 no. 31, Mortality Statistics (1998). [2] Multiple Risk Factor Intervention Trial Research Group. Relationship between baseline risk factors and coronary heart disease and total mortality in the Multiple Risk Factor Intervention Trial Prev. Med 1986; 15: 254-273. [3] Brindle JT, Antti H, Holmes E. et al Nature Medicine 2002; 8(12): 1439-1445. [4] National cholesterol education program report of the expert panel on the population strategies for blood cholesterol reduction. Circulation 1991; 83: 2154. [5] Thomassen AR, Neilson TT. Clin Sci. 1983; 64: 33-40. [6] Carlsten AB, Hallgren R, Jagenburg A et al. Scand J Clin Invest.13: 418-425. [7] Mudge GH, Mills RM, Taegtmeyer H et al. J Clin Invest. 1976; 58: 1185-1192.