Biochemical characterization of blood plasma of coronary artery disease patients by *in vitro* high-resolution proton NMR spectroscopy

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This study aimed to investigate the biochemical profile of blood plasma of patients with coronary artery disease (CAD) and angiographically normal subjects (controls) to determine biomarkers for their differentiation. In this double blind study, 5 mL venous blood was drawn before angiography from CAD patients (n=60) and controls (n=13) comprising angiography normal individuals. *In vitro* high-resolution NMR spectroscopy of these blood plasma samples was carried out at 400 MHz, and intensity data were analysed with partial least square discriminant analysis. Categorization of subjects as controls or CAD patients and the patients further as single vessel disease (SVD), double vessel disease (DVD) and triple vessel disease (TVD) was done at the end of the study based on their angiography reports. Raised levels of lipids, alanine (Ala) and isoleucine/leucine/valine (Ile/Leu/Val) were observed in CAD patients compared with controls. Partial least square discriminant analysis showed separation between controls *vs* CAD patients. TVD patients showed increased levels of Ile/Leu/Val and Ala compared with controls and SVD. Alanine, Ile/Leu/Val, and LDL/VLDL appear as possible biomarkers for distinguishing between controls and patients with SVD and TVD. A metabolic adaptation of myocardium may play a role in raising the Ala level.

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1. Introduction

Coronary artery disease (CAD) is a major cause of mortality and morbidity worldwide in spite of enormous progress made in prevention and treatment of the disease (Kochanek *et al.* 2011; James 2004). In urban population, the prevalence of CAD increased from 3.5% in the 1960s to 9.5% in the 1990s, while in rural areas it has increased from 2% in the 1970s to 4% by the 1990s in the Indian population (Gupta 2000). In general, CAD is associated with number of risk factors like increased plasma VLDL (very-low-density lipoprotein) and LDL (low-density lipoprotein) levels with decreased HDL (high-density lipoprotein) level (Kannel and Wilson 1995). High levels of cholesterol and LDL in blood leads to formation of plaques on the inner walls or lining of the arteries because of which coronary arteries become hardened and narrowed. The confirmatory test for diagnosis of CAD is coronary angiography, which is invasive.

To predict the risk of CAD, identification of molecules involved is determined by biochemical methods in addition to various lipid profile tests. High-resolution NMR spectroscopy of body fluids such as blood (Wevers *et al.* 1994), CSF (Kamlesh *et al.* 2005) and urine (Foxall *et al.* 1993) have

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proven to have the potential for identification of key molecules involved in various biochemical processes and have gradually developed as a major analytical technique (Wevers 1994). *In vitro* proton (^{1}H) MR spectroscopy (MRS) has also been used to investigate the metabolomics of various diseases such as prostate cancer (Cheng *et al.* 2001), Duchenne muscular dystrophy (Sharma *et al.* 2003), inflammatory bowel disease (Balasubramanian *et al.* 2009) and cardiac disease (Brindle *et al.* 2002). Earlier CAD studies reported in literature have focused only on the LDL/VLDL concentrations for the diagnosis by MRS (Blake *et al.* 2002). It is known that the number and the type of lipid particles present cause atherosclerosis, not the amount of cholesterol they carry. MR spectroscopy has the ability to measure the patient's lipoprotein particles, and the actual number of individual lipoprotein particles present thus can more accurately predict the cardiovascular disease than standard lipid testing. Further, the MR spectral profile of lipids can be used to determine LDL and its various subtypes and their concentration. This allows rapid diagnosis that may be useful to tailor therapies of individual patients (Otvos *et al.* 2002). Freedman *et al.* (1998) developed a ^{1}H MRS–based procedure for quantifying plasma levels of subclasses of VLDL, LDL and HDL. Moreover, a combination of ^{1}H MRS and sophisticated line-shape-fitting analysis method has been reported for the estimation of absolute concentration of lipoproteins in blood plasma (Ala-Korpela *et al.* 1994). Everett *et al.* (2005) reported a detailed study on seven lipid measures and found these to be superior predictors of CAD risk. These include total cholesterol, HDL cholesterol, LDL cholesterol, VLDL, triglycerides, apolipoprotein A-1 and apolipoprotein B. Bathen *et al.* (1999) have used principal component analysis (PCA) to study the lipoprotein fractions obtained by ultracentrifugation of blood plasma in CAD patients and controls.

In determining the severity of the disease, pattern recognition approach has also been applied to the MR data of CAD patients (Brindle *et al.* 2002). Kirschenlohr *et al.* (2006) have documented that ^{1}H MRS showed only weak discrimination of angiographically classified CAD patients. The objectives of the present preliminary study are (a) to determine a simple and fast approach using NMR, PCA and partial least squares discriminant analysis (PLS-DA) for classifying CAD patients; (b) to analyse the low-molecular-weight metabolites in blood plasma of patients and controls along with the lipid profile to understand the underlying metabolic changes of myocardial adaptations in CAD; and (c) to determine possible biomarkers that may be useful in differentiating the controls and different forms of CAD, namely, single vessel disease (SVD), double vessel disease (DVD) and triple vessel disease (TVD).

2. Materials and methods

2.1 *Sample collection and preparation*

This is a double blind study wherein 5 mL of venous blood was drawn from the subjects (n=73) before they underwent angiography at the Department of Cardiology. Controls comprised of angiography normal individuals (n=13; mean age 52.0±11.9 years; 5 mL blood was drawn from these subjects also before they were to undergo angiography). The mean age group of CAD patients recruited was 56.1 ±11.4 years.

The blood sample was collected in a tube containing heparin and centrifuged at 3000 rpm for 15 min at 4°C. The plasma was carefully isolated and stored at −35°C until NMR spectroscopy experiments, which were conducted within 24 h. Prior to carrying out NMR experiments, the blood plasma samples were thawed at room temperature. A total sample volume of 600 µL was used for NMR spectroscopy. This contained 420 µL of the plasma sample and 180 µL of 10 mM of sodium trimethyl-silyl-propionate (TSP) from stock solution made in D_2O, which makes the final concentration of TSP to be 3 mM in the solution used for NMR. The NMR experiments were carried out immediately at 298K. The institute ethics committee approved the study.

2.2 *NMR spectroscopy*

The ^{1}H MR experiments were performed using a narrow-bore DRX-400 (BRUKER) spectrometer operating at 400.13 MHz equipped with multinuclear broadband inverse probe. One-dimensional (1D) NMR spectrum with water suppression was acquired using the following parameters: 32K data points, spectral width of 4000 Hz, number of scans were 32 with a relaxation delay of 14 s. The FIDs were multiplied by an exponentially decaying function prior to Fourier transformation leading to a line broadening of 0.3 Hz. Spectra were manually phased using zero- and first-order corrections.

Two-dimensional (2D) double quantum filtered correlated spectroscopy (DQF-COSY) and total correlation spectroscopy (TOCSY) were acquired using standard software provided by the manufacturer. The typical parameters used for DQF-COSY were: 2K data points, spectral width of 4000 Hz, 512 experiments in t_1 dimension with 64 acquisition and 16 dummy scans to obtain steady state magnetization. The water resonance was pre-saturated during the relaxation delay of 2.5 s. The typical parameters used for TOCSY were: 1K data points in t_2 domain covering a spectral width of 4000 Hz. The water resonance was pre-saturated during the relaxation delay of 2.5 s.

2.3 *Data analysis*

The intensities of well-resolved isolated resonance of metabolites of interest were determined by integration of the NMR peak and were normalized with respect to the intensity of the TSP resonance (3 mM). Data were analysed using a statistical software program (SPSS version 11.5 for Windows: SPSS, Inc., Chicago, IL). The samples were divided into four groups namely, Controls (CON), SVD, DVD and TVD, based on their disease status and angiography reports. A one way analysis of variance (ANOVA) along with Bonferroni correction was used to compare the intensities of various metabolites among the groups. Probability value of 5% was recorded as significant ($p \leq 0.05$). PCA and PLS-DA (Brindle *et al.* 2002) was also carried out on the NMR intensity data of the metabolites to explore any clustering behaviour of the samples from CAD patients and controls using Unscrambler 10.2 (Camo, Oslo, Norway). PCA is an unbiased mathematical algorithm that lowers data dimensionality whilst retaining variation in a large dataset. By identifying directions (principal components) in which variation are at maximum, samples can be explained by a relatively low number of components instead of thousands of variables. Analysis of the components plots were used to identify similarities and differences between samples. The PLS is a supervised method that uses multivariate regression techniques to extract via linear combination of original variables (X) the information that can predict the class membership (Y). The data was arranged as a table in excel sheet with one sample per row and one variable (intensity of the metabolite) per column. The data was preprocessed using unit scaling (mean-centered and divided by standard deviation of each variable) method before PLS-DA analysis.

3. Results

3.1 *Spectral assignments*

The categorization of the subjects as controls or CAD patients and the patients further as SVD, DVD and TVD was done at the end of the study based on their angiography reports. Figure 1 shows the 1D proton NMR spectrum of the whole blood plasma sample from a CAD patient (TVD). The assignments of various metabolites were carried out using 1D and 2D NMR experiments like DQF-COSY and TOCSY (figure 2). In addition, the assignments of some of the metabolites were carried out using 1D spiking experiments. The proton NMR spectra of blood plasma samples showed peaks due to lipoproteins, amino acids and organic acids. Table 1 gives the values of the chemical shift positions of nineteen metabolites that were unambiguously identified

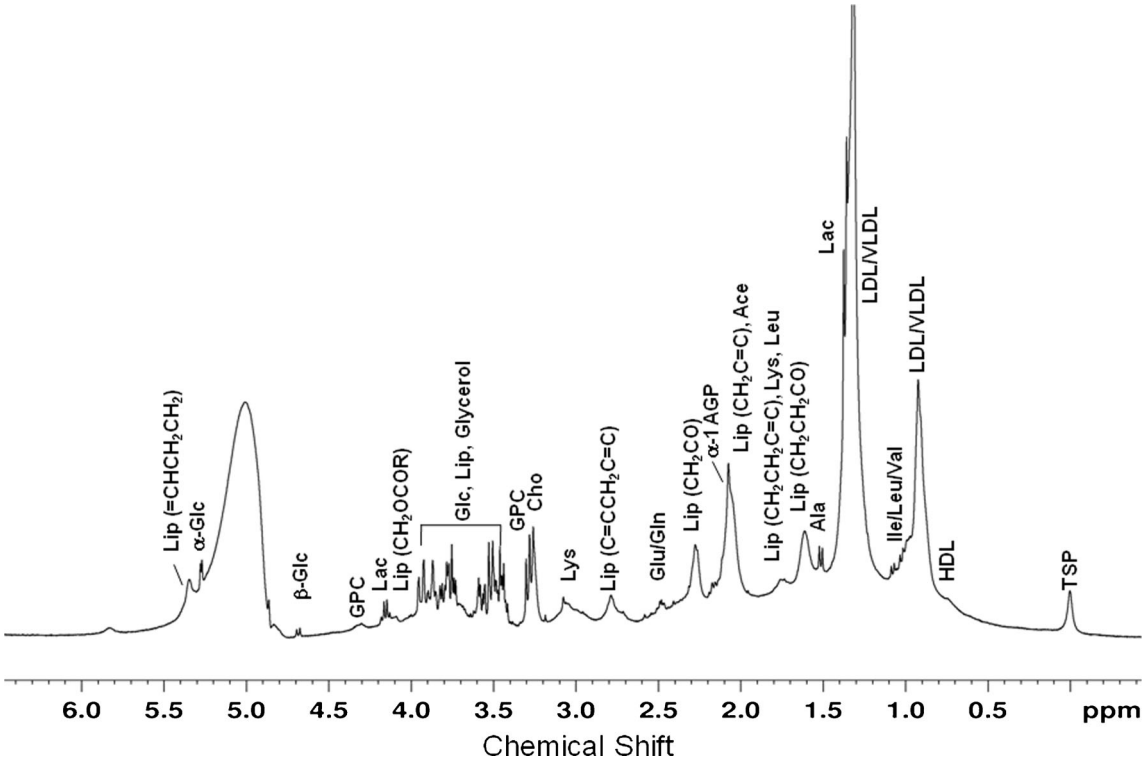

Figure 1. Representative expanded region from 0 to 6 ppm of 1D ^{1}H NMR spectrum recorded at 400 MHz of the blood plasma obtained from a TVD patient at 298K.

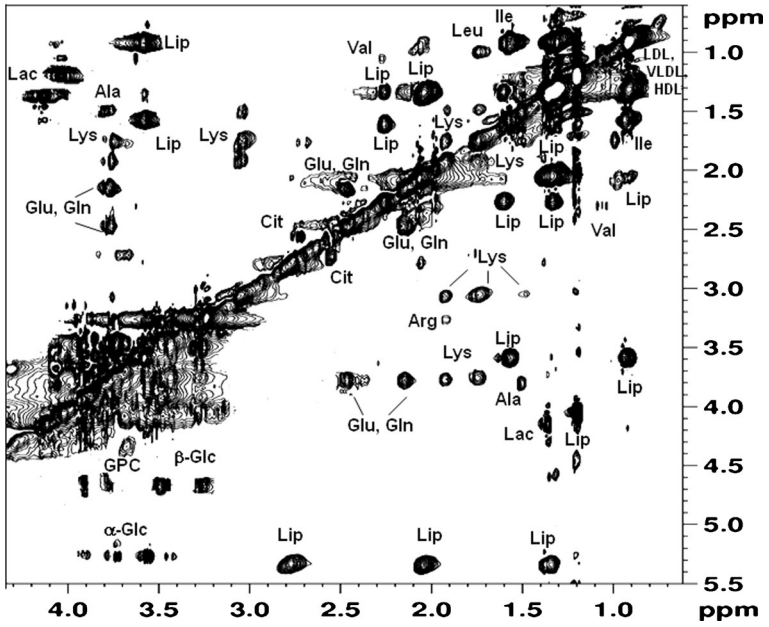

Figure 2. Representative expanded aliphatic region of the 2D total correlation spectroscopy (TOCSY) ^{1}H NMR spectrum at 400 MHz of the blood plasma obtained from a TVD patient recorded at 298K.

in the NMR spectra of blood plasma and were in agreement with that reported in literature (Nicholson and Foxall 1995; Balasubramanian *et al.* 2009).

The methyl protons of leucine (Leu), isoleucine (Ile), and valine (Val) appear between 0.90–1.00 ppm. The other protons of Ile, Val and Leu were assigned using cross peaks observed in DQF-COSY. The methyl protons of Ala at 1.48 ppm showed connectivity to its α-CH at 3.75 ppm. The CH_2 protons of arginine (Arg), Leu and lysine (Lys) were assigned to the peaks between 1.72-1.8 ppm. The resonances due to CH_2 protons of glutamate (Glu) and glutamine (Gln) appear as multiplet at 2.36 ppm. The triplet of taurine (Tau) contributed by CH_2NH protons was confirmed by spiking experiment at 3.24 ppm.

The assignments of resonances from few organic acids were also carried out in the blood plasma samples. A cross peak was seen between the methyl protons at 1.33 ppm and α-CH proton of Lac at 4.10 ppm in TOCSY and DQF-COSY confirming the assignment of Lac tate (Lac). A singlet peak at 1.91 ppm was assigned to the methyl protons of acetate (Ace). The H1 of α-glucose (Glc) was assigned at 5.23 ppm which showed coupling to its H2 proton at 3.54 ppm in DQF-COSY and TOCSY. Similarly the H1 of β-Glc was observed as a doublet at 4.64 ppm and the assignment was confirmed via its connectivity with H2 at 3.26 ppm.

Several resonances such as methyl, methylene, allyllic and olefinic protons from different lipids (Lip) and triglycerides were also seen in the MR spectrum of the blood plasma. The resonances from the terminal CH_3 protons of LDL and VLDL overlap at 0.90 ppm. An intense cross peak was observed between CH_3 and CH_2 protons of LDL and VLDL in DQF-COSY and TOCSY (figure 2). The resonance corresponding to CH_3 of HDL was assigned at 0.84 ppm. The acetyl groups of N-acetyl glucosamine and N-acetyl neuraminic acid of α-1 acid glycoprotein (α-1 AGP) contribute to a peak at 2.06 ppm.

3.2 *Analysis of intensities of metabolites obtained in whole blood plasma*

The intensities of various metabolites were normalized to the intensity of TSP. Table 2 shows the mean normalized intensity with the standard deviation between angiography normal (controls) and CAD patients. Statistically significant differences were observed in various metabolites across the groups of patients and controls. The total sample size analysed was seventy-three which included angiography normal (control, n=13); SVD (n=20); DVD (n=19) and TVD (n=21) samples.

An elevated level of LDL/VLDL was observed in TVD patients compared to patients with SVD (table 2; figure 3). A significant increase in branched chain amino acids like Ile/Leu/Val was observed between the two groups namely, controls *vs* TVD and SVD *vs* TVD. However, the other groups did not demonstrate any significant change. Similarly, an increase in the intensity of Ala was observed in TVD patients compared to patients with SVD and controls (table 2; figure 3). However, other metabolites such as α-1 AGP, Lac, Glu and Glc showed

Table 1. The chemical shift positions of various metabolites observed in the blood plasma in the ^{1}H NMR spectrum of a patient with triple vessel disease (TVD)

Metabolite	Chemical shifts (ppm)
Leucine (Leu)	0.97; 0.95 (δ-CH_3), 1.69(γ-CH), 1.72 (β-CH_2), 3.69 (α-CH)
Isoleucine (Ile)	0.92; 0.93 (δ-CH_3), 1.25, 1.47(γ-CH_2), 1.96 (β-CH), 3. 75 (α-CH)
Valine (Val)	1.00 (γ-CH_3), 2.24 (β-CH)
Alanine (Ala)	1.48 (β-CH_3), 3.75 (α-CH)
Glutamate (Glu)	2.04(β-CH_2), 2.36 (γ-CH_2), 3.65 (α-CH)
Glutamine (Gln)	2.08 (β-CH_2), 2.34 (γ-CH_2), 3.66 (α-CH)
Arginine (Arg)	1.68 (γ-CH_2), 1.91 (β-CH_2), 3.24 (δ-CH_3), 3.63 (α-CH)
Lysine (Lys)	1.72 (δ-CH_2), 1.91(β-CH_2), 3.01 (ε-CH_2), 3.63 (α-CH)
Lactate (Lac)	1.33(β-CH_3), 4.10 (α-CH)
Acetate (Ace)	1.91 (β-CH_3)
Choline (Cho)	3.20 [N (CH_3)$_3$]
Citrate (Cit)	2.56 (1/2CH_2), 2.74 (1/2CH_2)
α-Glucose (α-Glc)	5.22 (H1), 3.53 (H2), 3.71(H3), 3. 41 (H4), 3.84 (H5), 3.72 (CH_2)
β-Glucose (β-Glc)	4.64 (H1), 3.24 (H2), 3.40 (H4), 3.47 (H5) 3.48 (H3), 3.72 (CH_2)
Glycerophosphocholine (GPC)	3.23 (NCH_3), 3.69 (NCH_2), 4.31 (H4)
β-hydroxy butyrate (β-HB)	1.20 (γ-CH_3), 4.3 (β-CH), 2.31 (α-CH_2)
α-1Acid Glycoprotein (α-1AGP)	2.06 (CH_3)
Low-density lipoprotein (LDL)	0.90 (CH_3), 1.30 (CH_2)$_n$
Very-low-density lipoprotein (VLDL)	0.90 (CH_3), 1.30 (CH_2)$_n$
High-density lipoprotein (HDL)	0.84 (CH_3)
Lipid (Lip)	0.93 (CH_3CH_2), 1.32 ($CH_2CH_2CH_2CO$), 1.57 (CH_2CH_2CO), 1.62 (CH_2CH_2C=C), 2.04 (CH_2C=C), 2.25 (CH_2CO), 2.76 (C=CCH_2C=C), 4.06 (CH_2OCOR), 5.33 (=$CHCH_2CH_2$)

no significant difference among the different groups of CAD patients and controls.

3.3 *Multivariate analysis*

The PLS-DA scores plot of the first and second factors showed clustering of controls and TVD with the exception of patient # 6 of TVD which clustered with the control group (figure 4A). The R2 was 0.67 and Q2 was 0.61 for the PLS model for controls *vs* TVD patients indicating the validity of model. Similarly a clear separation between control and DVD was evident, however, the data of one control #N2 was found in the cluster of DVD (R2= 0.65; Q2=0.62) (figure 4B). A separation was also evident between SVD *vs* TVD (R2= 0.59; Q2=0.57) and SVD *vs* DVD (R2= 0.55; Q2=0.52) with the formation of separate cluster, however with some overlap (figure 4C and D).

4. Discussion

Blood is a heterogeneous mixture of lipoproteins, proteins, small organic molecules and ions. In the present study, *in vitro* 1D and 2D proton MR spectroscopy methods were used to characterize the biochemical profile of blood plasma of the patients with CAD. As stated earlier, this was a double blind study and the categorization of the subjects as controls or CAD patients and the patients further as SVD, DVD and TVD was done at the end of the study based on their angiography reports. The MR data were compared with angiography of normal controls to understand the metabolism of CAD patients and obtain biomarkers for the differentiation of the disease from controls. In addition, PCA and PLS-DA was also carried out on the NMR intensity data of the metabolites to explore the clustering behaviour of the samples from CAD patients.

The PLS-DA scores plot of the first and second factor showed clustering of samples of controls and TVD with marginal overlap. Similarly, some overlap was seen in the samples of controls and DVD, although separate clusters were evident. In general, the most influential metabolites for the separation for DVD and TVD samples from control were primarily LDL and VLDL, Ile/Leu/Val and Ala as observed in univariate analysis. These are in agreement with the previous studies (Brindle *et al.* 2002). The MR data of the whole blood plasma showed increased intensity of LDL/VLDL in patients with TVD in comparison with patients with SVD. Stamler (1984) reported that for every 1% increase in total cholesterol, the risk for CAD rises by 2% through epidemiological studies. This relationship was supported by clinical trials (see 1984 Lipid Research Clinics Program) and it has been shown that 1% reduction in the cholesterol level reduces the risk of CAD by 2%, and the relationship is known as 1–2% rule.

The interesting finding of the present study was the statistically significant increase in Ala levels in the whole blood plasma of TVD patients compared with angiography normal subjects and SVD patients. Since the Ala peak is close to lipid resonance, it is possible that the baseline around Ala peak may get affected (Brindle *et al.* 2002; Kirschenlohr *et al.* 2006), which may lead to systematic difference in the accuracy of measurements of Ala in whole blood plasma. In order to check whether the increase in the metabolites like Ala and Ile/Leu/Val is due to severity of disease, NMR of few blood plasma samples from angiography normal controls, SVD, DVD and TVD after perchloric acid extraction was carried out. The concentration was estimated using TSP

Table 2. Comparison of intensities of various metabolites present in whole blood plasma determined from 1D ^{1}H NMR in controls (CON) and patients with SVD, DVD and TVD using one-way ANOVA test with Bonferroni correction

Metabolites	Controls (a) (n=13)	SVD (b) (n=20)	DVD (c) (n=19)	TVD (d) (n=21)	p-value, ANOVA	F value ANOVA	*Post hoc* p<0.05 between groups
LDL/VLDL	31.7±6.0	32.0±3.7	37.7±7.3	39.2±8.7	0.015	3.89	(b, d)
Ile/Leu/Val	18.9±4.0	19.4±3.5	22.2±4.9	24.2±3.4	0.008	4.42	(a,d), (b,d)
Ala	5.6±1.0	6.7±1.9	8.4±2.7	9.5±3.5	0.002	5.73	(a,d), (b,d)
α-1 AGP	14.2±3.0	16.7±3.8	18.0±6.0	18.4±6.0	0.206	1.57	NS
Lac	3.9±0.8	3.9±1.0	4.5±1.2	4.4±1.1	0.319	1.19	NS
α-Glc	3.8±1.4	4.4±1.2	5.0±1.6	4.8±1.8	0.201	1.58	NS
β-Glc	0.45±0.2	0.82±1.1	0.79±0.5	0.79±0.5	0.510	0.78	NS
Glu+Gln (Glx)	0.40±0.08	0.45±0.11	0.57±0.25	0.50±0.21	0.244	1.45	NS

Abbreviations used: SVD, single vessel disease; DVD, double vessel disease; TVD, triple vessel disease; LDL, low-density lipoproteins; VLDL, very-low-density lipoproteins; Ala, alanine; Ile/Leu/Val , isoleucine/leucine/valine; α-1 AGP, alpha-1-glycoprotein; Lac, lactate; Glc, glucose; Glu+Gln (Glx); glutamate+glutamine; NS: not significant.

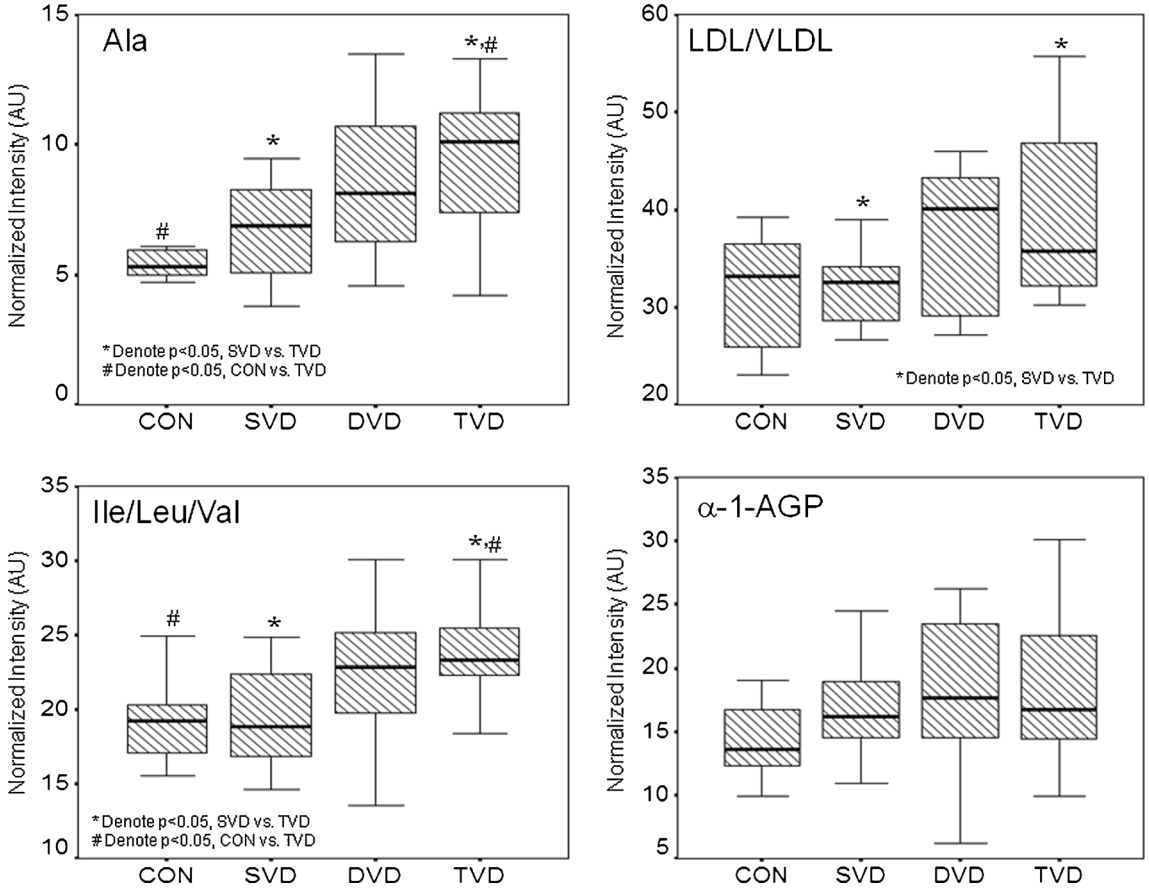

Figure 3. Box plots showing the comparison of normalized intensity obtained from 1D proton NMR spectrum of lipoproteins LDL/VLDL, isoleucine/leucine/valine (Ile/Leu/Val), alanine (Ala) and alpha-1 glycoprotein (α-1 AGP) in angiogrphically normal (CON) subjects and patients with SVD, DVD and TVD.

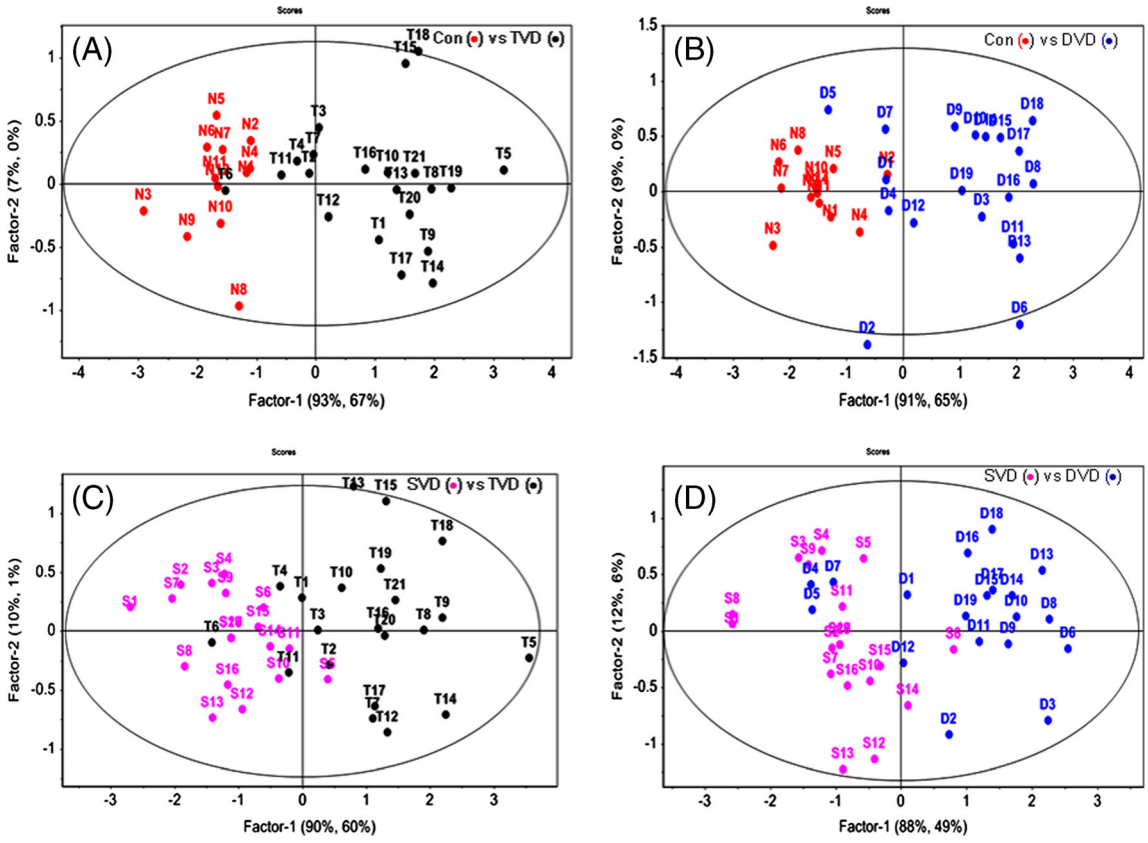

Figure 4. PLS-DA scores plot showing the considerable separation between (**A**) controls, CON (N ●) *vs* TVD (T•); (**B**) controls, CON (N ●) *vs* DVD; (D ●); (**C**) SVD (S ●) *vs* TVD (T•); (**D**) SVD (S ●) *vs* DVD (D ●).

as a reference as described in the literature (Sharma *et al.* 2003; Balasubramanian *et al.* 2009). The concentration of Ala and Ile/Leu/Val showed increasing trend as a function of severity of the disease. Statistically significant increase was observed in patients with TVD compared to controls.

In CAD patients, Ala release has been shown to be related to the severity of coronary artery stenosis (Thomassen *et al.* 1983). It has been reported that there is no net uptake and release of amino acids by the human heart except Glu and Ala (Carlsten *et al.* 1961). Ala is a dietary non-essential gluconeogenic amino acid and is synthesized in muscle by transamination of pyruvate and then released into the blood stream. Liver takes up Ala from the blood and its carbon skeleton is reconverted to glucose through the Cori Cycle. Glucose is then released into the blood stream. Glutamate also plays a special role in the interrelation of metabolic pathways in myocardial cells, which is evident by its high concentration in cardiomyocytes (Scharff and Wool 1965). Pisarenko *et al.* (1989) showed that the major metabolic branch point linking amino acid and carbohydrate metabolism is the transaminase reaction which catalyses the conversion of pyruvate to Ala and alpha ketoglutarate to Glu.

This amino acid exchange may be beneficial to the ischemic cell in several ways, such as Ala may act as a non-toxic carrier of ammonia that accumulates rapidly during anoxia. In addition, the conversion of pyruvate to Ala instead of Lac may reduce the accumulation of Lac and thereby during ischemia may help relieving inhibition of glycolysis. Both during aerobic and anaerobic conditions, the amino acids may act as fuel, but the energy production from amino acid remains negligible compared with that from glycolysis. The most important advantage of amino acid exchange process is that it leads to the transport of reducing equivalents across the mitochondrial membrane to reoxidize cytosolic nicotinamide adenine dinucleotide via the malate-aspartate cycle. The malate-aspartate cycle is essential for Glc and Lac breakdown in heart and is dependent on the presence of Glu (Scharff and Wool 1965; Pisarenko *et al.* 1989).

During pacing stress, Ala is formed in normal myocardium from Glu but not at rest. However, it has been reported that Ala was released both during pacing stress and at rest in diseased heart (Mudge *et al.* 1976). Our study has shown a significant increase in Ala concentration in TVD patients.

These observations suggest the hypothesis that chronic or repetitive bouts of myocardial ischemia may induce alterations in myocardial amino acid metabolism. The enhanced uptake of Glu and the release of Ala noted in the CAD patients are thus suggestive of a metabolic adaptation of the myocardium to chronic ischemia. Thomassen *et al.* (1983) have shown that for assessing myocardial ischemia, the measurement of myocardial exchange of Glu and Ala may serve as a sensitive biochemical test. However, in the present study, no significant difference was observed in the intensity of Glu + Gln among controls and various groups of CAD.

Further, a significant increase in Ile/Val/Leu amino acids was observed in TVD patients compared with the controls and patients with SVD. It was reported that the branched-chain amino acids, namely Ile and Val, as well as amino acids like aspartate and Glu, provide the carbon skeleton for citric acid (Gibala *et al.* 2000). An increase in levels of Leu in blood leads to an increase in protein degradation as well as synthesis in a mouse model deficient of the mitochondrial branched chain aminotransferase (Fried and Watford 2007; She *et al.* 2007). Taegtmeyer *et al.* (2008) reported that metabolic signaling from micronutrients regulate the protein turnover and proposed that the supplementation of branched-chain amino acids may aid in repair of the diseased heart. Therefore, it may be hypothesized that the increased levels of Ile/Leu/Val observed in the present study may be indicative of the myocardial adaptations and mechanisms developed for rejuvenating the ischemic injury of heart.

There are a few limitations of this study. The first limitation is the small number of samples were used in the present study. Secondly, even though studies have used TSP as an internal standard (Ruiz-Calero et al 2002; Cartigny *et al.* 2008), few studies have reported that TSP is adsorbed by plasma proteins leading to reduction in estimation of TSP concentration using NMR (Kriat *et al.* 1992; Ando *et al.* 2010). In view of this, the absolute concentration of metabolites of the plasma samples was not determined in the present study. To achieve this formate needs to be used as concentration standard, but a longer relaxation delay should be used (>30 s) as the T1 of formate is long (Ando *et al.* 2010). However, the distinction between TVD from SVD and controls are clearly evident using relative intensity data shown in the present study. Another limitation is the presence of macromolecules that may lead to baseline distortions and broadening of metabolite signals in 1D spectrum using the pulse-acquire sequence of plasma samples. The use of Car-Purcel-Meiboom-Gill (CPMG) spin echo experiments may alleviate the baseline distortions and broadening due to macromolecule resonances and may provide a more appropriate quantification. Thus, further studies are required with formate as an internal concentration standard using CPMG sequence and such a study is in progress in a large cohort of CAD patients.

5. Conclusion

In conclusion, our data, albeit less in number, clearly indicated that it is possible to identify CAD patients, especially TVD patients from controls and patients with SVD by NMR metabolomics of blood plasma. The statistical analysis using ANOVA demonstrated significant increase in Ala, Ile/Leu/Val as well as LDL/VLDL in patients with TVD compared with patients with SVD and controls. Shunting of pyruvate to Ala instead of Lac would relieve inhibition of glycolysis during ischemia, thereby suggesting a metabolic adaptation of the myocardium to CAD. Studies are underway at higher magnetic fields in a large cohort of CAD patients to further substantiate our interesting finding of establishing Ala and Leu/Ile/Val as biomarkers for distinguishing between patients with SVD, DVD and TVD using formate as the standard.

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