6.1 Introduction

"Biomarker" has become a buzzword in life sciences. Building fundamental science, supporting early diagnosis of diseases and following their progression, improving efficacy and safety of treatments, optimizing patient selection and helping decide which therapy is most appropriate; these are examples of a few contexts in which biomarkers are key players. Briefly, today's biomarkers are the predictors of tomorrow's therapies. Recently Doorn M et al (2007) reported that the NMR-based metabolomics of urine and blood plasma samples can yield a broad array of early responding biomarkers for the effects of rosiglitazone in T2DM patients, as well as nonglucose biomarkers that may reflect the T2DM state. Recently Srinubabu et al (2007) reviewed the importance of bioinformatic tools for identification of biomarkers in T2DM and its complications. So new technologies such as metabolomics, proteomics and bioinformatics are do necessary for better therapeutic interventions.

Bioinformatics has been in the focus since recent years for unraveling the structure and function of complex biological mechanisms. The analysis of primary gene products has further been considered as diagnostic and screening tool for disease recognition. Such strategies aim at investigating all gene products simultaneously in order to get a better overview about disease mechanisms and to find suitable therapeutic targets. Our analysis is focused on potential implications of bioinformatics as a tool to identify novel metabolic patterns or markers associated with disease status by taking DMC as an example. In the present in silico study we have employed clustalW online
bioinformatics tool for the analysis of genes, which are expected to play major role in DMC, we sought to identify the common central gene/protein that connects both the metabolic disorders such as obesity and diabetes.

6.2 Results & Discussion

Diabetic Cardiomyopathy

The main forms of structural heart disease associated with diabetes are coronary heart disease and diabetic cardiomyopathy, which is characterized by left ventricular hypertrophy, left ventricular diastolic and systolic dysfunction. Asymptomatic structural heart disease is common and associated with a poor prognosis in patients with diabetes (Somaratne JB, 2008).

The results of our bioinformatics analysis showed that Na+-Ca2+-exchanger, protein kinase C-β, and Na+-K+-ATPase as the key proteins that are closely associated with diabetic cardiomyopathy out of eight data mining proteins (table 6.1). Proteins with minimum distance are Na+-Ca+2-exchanger, protein kinase C-β isomer and Na+-K+-ATPase suggesting that these are not only closely linked with each other but also play a significant role in the pathobiology of diabetic cardiomyopathy (figure 6.1).

Long standing diabetes can produce myocardial contractile dysfunction even in the absence of CAD and hypertension (Uusitupa M.et.al.1990). The most typical feature of diabetic cardiomyopathy is the abnormal filling pattern of left ventricle with reduced compliance or prolonged relaxation (Penpargkul S.et.al.1981). The shift in the faster V1 form of myosin to slower V3 form leads to delayed relaxation of the ventricles. Predominance of myosin subtype V3 reduces the Ca2+-ATPase activity (Lazar HL.et.al.2006). Impaired sarcoplasmic Na+-Ca2+ exchanger activity and depressed Na+-
K+-ATPase activity causes retention of calcium that could render myocardial contractile
dysfunction.

In addition, diabetic cardiomyopathy may be associated with aberrations in
glucose and lipid metabolism (Guo M. et al. 2003), which are known to occur in patients
with diabetes mellitus, that could lead to secondary to disturbances in carbohydrate, lipid
and adenine nucleotide metabolism in the diabetic heart (Ligeti L. et al. 2006). In
particular, depletion of glucose transporter-4 (GLUT-4), increase in fatty acids, changes
in calcium homeostasis, and associated small vessel disease, cardiac autonomic
neuropathy, and insulin resistance may all play a significant role in the onset of diabetic
cardiomyopathy.

It was reported that decreased sarcoplasmic reticulum Ca2+-ATPase activity
results in decreased calcium transport in isolated sarcoplasmic reticulum obtained from
animals and humans with diabetes mellitus. Furthermore, other abnormalities of Ca2+
homeostasis that could occur in these patients include: i) decreased Ca+2 uptake, ii)
decreased Ca2+ binding, and iii) decreased Na+-K+-ATPase activity of sarcolemma
(Ligeti L. et al. 2006). This is supported by the observation that sarcolemmal and the
sarcoplasmic reticular calcium transporters are depressed in diabetic cardiomyopathy.
This results in an increase in cytoplasmic calcium content and a decrease in calcium
outward current resulting in prolonged action potential duration and increased myocardial
stiffness. This altered intracellular calcium handling eventually leads to decreased

Transgenic mice overexpressing PKC-β isoform developed cardiac hypertrophy
and myocardial fibrosis, whereas PKC-β isoform inhibitor prevented several of the
functional abnormalities seen in diabetic cardiomyopathy (Wakasaki H.et.al.1997). This is supported by the observation that activation of PKC can modulate the gene expression of the myocardium that results in myocardial hypertrophy and myocardial fibrosis and eventually causes myocardial failure (Tschope C.et.al.2004). Since PKC appears to be involved in the pathobiology of development of several complications seen in diabetes mellitus and as diabetic state itself induces the activation of PKC-β isoform that can produce cardiac abnormalities (as evidenced from the studies done with Transgenic mice overexpressing PKC-β isoform), the involvement of PKC in diabetic cardiomyopathy appears persuasive.

It is known that accumulation of myocardial collagen resulting in interstitial and perivascular fibrosis that occurs in long-standing diabetes can be correlated with early diastolic and systolic left ventricular dysfunction (Iwata K.et.al.2006). Non-enzymatic glycosylation of collagen and diminished activity of collagen degrading enzymes such as matrix metalloproteinases (MMP) are considered to be pathognomonic of myocardial fibrosis (Iwata K.et.al.2006) that could eventually lead to myocardial dysfunction in diabetes. There is also general consensus that formation of advanced glycation end products that occurs as a result of persistent hyperglycemia can also cause cellular and myocardial dysfunction in diabetes (Bidasee KR.et.al.2004, Pacher P.et.al.2002).

In addition, there is evidence to suggest that activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) contributes to the development of endothelial and myocardial dysfunction in diabetes. It was reported that hyperglycemia, cardiac PARP activation, a selective loss of endothelium-dependent vasodilation in the thoracic aorta, and an early diastolic dysfunction of the heart accompanied development of diabetes in
experimental animals (Kiss L.et.al.2005, 13). Treatment with PARP inhibitor, starting 1 week after the onset of diabetes, restored normal vascular responsiveness and significantly improved cardiac dysfunction, despite the persistence of severe hyperglycemia. The beneficial effect of PARP inhibition persisted even after several weeks of discontinuation of the treatment. Thus, PARP activation plays a central role in the pathogenesis of diabetic cardiovascular (cardiac as well as endothelial) dysfunction and PARP inhibitors may exert beneficial effects against the development of cardiovascular complications in diabetes.

It is evident from the preceding discussion that several pathological processes are proposed and seems to be involved in the development of diabetic cardiomyopathy. Of all these mechanisms, the earliest pathological event appears to be altered calcium homeostasis. The results of the present bioinformatics study also support the role of PKC in the development of cardiac abnormalities seen in diabetic cardiomyopathy. Thus, our bioinformatics study highlights the involvement of deranged calcium homeostasis and impairment of contractile proteins as the major responsible for the development of diabetic cardiomyopathy.

**Diabetic Coronary Artery Disease**

The results of our bioinformatics analysis showed that adiponectin and nitric oxide are the key proteins that are closely associated with diabetic coronary artery disease out of nine data mining proteins (table 6.2). Proteins with minimum distance are adiponectin and nitric oxide suggesting that these are not only closely linked with each other but also play a significant role in the pathobiology of diabetic coronary artery disease (figure 6.2).
Nitric oxide acts as anti atheroscleotic molecule that is synthesized from different isoforms of NO synthase (NOS). Endothelial dysfunction resulted from decreased production and/or bioactivity of nitric oxide and impaired endothelial dependent vasodilatation. Endothelial dysfunction is a major pathological event that links obesity, diabetes, and cardiovascular diseases. (Hattori Y. et al. 2003)

Few studies reported that insulin resistance aggravates endothelial dysfunction and several studies suggest that endothelial dysfunction contributes to insulin resistance. Majority of the reports suggest that endothelial dysfunction is an early event in the insulin resistance syndrome. Insulin is a vasodialater that promotes the expression of eNOS through the phosphatidyl-inosital-3-kinase (PI3K) pathway. This effect of insulin is blunted in patients with insulin resistance. Perturbations in insulin mediated glucose uptake or majorly attributed to endothelial dysfunction. Free fatty acids and proinflammatory adipokines are also causing endothelial dysfunction in the state of insulin resistance. (Willa A. et al. 2003). Endothelial dysfunction results in impaired insulin action by altering the transcapillry passage of insulin to target tissues (Sydow K. et al. 2005)

Previous represented that -786T C mutation leads to a pronounced impairment of vasodilation that could cause impaired insulin mediated glucose uptake throughout the body. (Ohtoshi K. et al. 2002)

Globular adiponectin up regulates the gene expression of eNOS in vascular endothelial cells. Adiponectin mimics the vascular and metabolic actions of insulin. Thus adiponectin also enhances glucose disposal by stimulating the production of nitric oxide
In vascular bed. In insulin resistance state a low adiponectin levels further influence the eNOS activity. (Hattori Y.et.al.2003)

In addition, adiponectin reduces the expression of adhesion molecules in endothelial cells and decreases cytokine production from macro phages. (Seung Hwan Han.et.al.2007) Adiponectin may also contributes to anti-atherogenic properties.

Insulin resistance is associated with the abnormalities that can alter endothelial function, such as hyperglycemia, hypertension, dyslipidemia. (Enrique Caballero.et.al.2003). Novel therapeutic approaches that improves endothelial function ameliorates insulin resistance where as improving insulin sensitivity ameliorates endothelial dysfunction. Previous studies strongly support a reciprocal relationship between endothelial dysfunction and insulin resistance that helps to link cardiovascular and metabolic diseases. (Jeong-a.et.al.2006) Peroxisome Proliferator Activator Receptor-gamma (PPAR-gamma) is expressed in endothelial cells and its ligands have been reported to improve insulin sensitivity and endothelial function. (Willa A.et.al.2004)

Type II. Diabetes its regarded as in a state of insulin resistance, In diabetes, sustained hyperglycemia causes increased intracellular concentrations of glucose metabolites in endothelial cells. All these changes cause mitochondrial dysfunction, enhanced oxidative stress and stimulation of Protein Kinase C. Consequently all these sequence of events produces endothelial dysfunction. (Rask-Madsen.et.al.2007)

Adiponectin is an adipocyte derived hormone with profound insulin sensitizing, anti-inflammatory and antiatherogenic affects. It is also associated with both insulin resistance and atherosclerosis. Decreased plasma adiponectin and insulin resistance coexist in subjects with prediabetes, diabetes and atherosclerosis. Adiponectin is an
independent correlate of insulin resistance and atherosclerosis. Adiponectin could be used as a biomarker to assess prediabetic state and atherosclerosis. (Subhashini. et al. 2006)

**Diabetes and Stroke**

The results of our bioinformatics analysis showed that Angiotensin-converting enzyme (ACE) is the key protein out of eleven data mining proteins (table 6.3). ACE is not only closely linked with each other but also play a significant role in the pathobiology of diabetic with stroke. Angiotensin-converting enzyme (ACE) polymorphism may play a role in stroke and silent brain infarction (SBI) susceptibility, but the results among the populations studied to date have not been consistent. There was a significant association between ACE polymorphism and ischemic stroke in the Asian population. Although no consistent associations have been found between ACE polymorphism and stroke in the populations studied to date, the ACE polymorphism may be a genetic determinant of ischemic stroke, at least in Korean patients (Hong SH. et al. 2007). Data concerning an association between angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism and ischemic stroke (IS) remain inconsistent. Few studies reported there is no association between ACE polymorphism and etiological subtypes of IS in a Polish population. (Pera J. et al. 2006)

Despite leading cause of morbidity and mortality in the United States. Strong evidence from twin and family studies shows that familial predisposition, in addition to such recognized risk factors as high blood pressure, smoking, diabetes, obesity, and advanced age, contributes to the pathogenesis of stroke. Identification and characterization of gene variants that play such a role may allow improved prognostication, therapy, and prevention. A polymorphic marker associated with the gene encoding ACE has attracted widespread attention
in recent years. This deletion/insertion (D/I) variant has consistently been shown to be associated with differential plasma and tissue ACE activities (Rigat B.et.al.1990, Tiret L.et.al.1992) its possible association with cardiovascular disorders, however, remains inconclusive. An association between the D allele and stroke incidence has been reported by some, but not by others. (Sharma P.et.al.1994)

**Diabetes and Peripheral Arterial Disease**

The results of our bioinformatics analysis showed that fibrinogen is the key protein out of four data mining proteins (table 6.4). Fibrinogen is not only closely linked with each other but also play a significant role in the pathobiology of diabetic with peripheral arterial disease.

There is conflicting evidence about the influence of fibrinogen genotype on plasma fibrinogen concentrations, Yet, certain fibrinogen genotypes are associated with an increased risk of peripheral atherosclerosis. 121 subjects with peripheral arterial disease and 126 healthy controls matched for age and sex were selected from a random population sample aged 55-74 years in the Edinburgh Artery Study. Mean fibrinogen concentrations were higher in cases than in controls (3.12 [95% confidence interval 2.99-3.26] vs 2.75 [2.64-2.85], p less than 0.001). A greater proportion of cases than controls were homozygous or heterozygous for an allele at the beta fibrinogen locus (4.2 kb allele, Bcl I digestion); the allele frequency was 0.197 in cases and 0.097 in controls (p less than 0.005). Extended haplotypes for 4.2 kb heterozygotes were also associated with an increased risk of peripheral arterial disease. However, haplotype had only a small effect on the association of plasma fibrinogen concentration with disease, and the relation of haplotype with disease was independent of age, sex, social class, smoking status, plasma fibrinogen, alcohol consumption, body mass index, and diabetes mellitus. Variation at
the beta fibrinogen locus is associated with an increased risk of peripheral atherosclerosis. The influence is not mediated simply by way of increased fibrinogen concentrations but could be due to a structurally variant fibrinogen or linkage disequilibrium with a neighbouring gene. (Fowkes FG. et al. 1992)

Genetic variation in plasma fibrinogen and the platelet receptor GP IIIa locus has been independently associated with increased risks of ischaemic heart disease. In contrast to previous findings, there was no significant relationship between fibrinogen T/G(+1689) genotype and ischaemic and peripheral heart disease in this older population. (Smith FB. et al. 2003)

As part of the five-year follow-up of the Edinburgh Artery Study, polymorphisms of the fibrinogen (-455G/A), factor VII (R/Q353) and PAI-1 (HindIII) genes were measured in men and women aged 60-79 years, together with their plasma levels. Using widely accepted criteria, 88 subjects were identified as having peripheral arterial disease (PAD), 195 having coronary artery disease (CAD) and 423 subjects comprised a "healthy" group. The -455AA genotype of the fibrinogen gene was found to be more frequent among those subjects with PAD. This genotype also showed the highest plasma fibrinogen levels in both disease groups and in the healthy group. Using logistic regression, after adjustment for age, sex, smoking and plasma level, the -455AA genotype was associated with over twice the risk of PAD compared with the -455GG genotype, the odds ratio reaching marginal significance (p < or = 0.10). Combining those with genotype -455AA with the heterozygotes in order to increase the power of the study resulted in a more significant multiple-adjusted risk of PAD (p < or = 0.05). These data provide evidence that a polymorphism of the P fibrinogen gene is associated with an increased risk of
peripheral atherosclerosis.(Lee AJ. et al. 1999). Moreover, the ACE I/D polymorphism was associated with PVD in these Type 2 diabetic patients.(Thomas GN. et al. 2003)

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Gene Name</th>
<th>Ac.No.</th>
<th>Length (amino acids)</th>
<th>Tissue Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Na⁺⁻K⁺⁻ATPase alpha-subunit</td>
<td>AAA35573.1</td>
<td>89</td>
<td>Placenta, brain</td>
</tr>
<tr>
<td>2</td>
<td>Na⁺⁻Ca²⁺ exchanger</td>
<td>CAA62923</td>
<td>40</td>
<td>airway smooth muscle</td>
</tr>
<tr>
<td>3</td>
<td>GLUT-4 transporter</td>
<td>NP_001033</td>
<td>509</td>
<td>NOT SPECIFIED</td>
</tr>
<tr>
<td>4</td>
<td>MMP2</td>
<td>ABD38929</td>
<td>61</td>
<td>NOT SPECIFIED</td>
</tr>
<tr>
<td>5</td>
<td>Protein kinase C, beta</td>
<td>NP_002729</td>
<td>673</td>
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</tr>
<tr>
<td>6</td>
<td>PPAR alpha</td>
<td>AAB32649</td>
<td>468</td>
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</tr>
<tr>
<td>7</td>
<td>p38 mitogen-activated kinase</td>
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<td>365</td>
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<tr>
<td>8</td>
<td>CD36</td>
<td>CAA83662</td>
<td>472</td>
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Table 6.1. Table showing the genes/proteins that have been studied in the present study, which are believed to be involved in diabetic cardiomyopathy.

<table>
<thead>
<tr>
<th>S.no</th>
<th>Gene Name</th>
<th>Accession Number</th>
<th>Length</th>
<th>Tissue</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>ACE</td>
<td>AAH36375</td>
<td>739 aa</td>
<td>Testis</td>
</tr>
<tr>
<td>2</td>
<td>ADIPOQ</td>
<td>AAH54496</td>
<td>244 aa</td>
<td>Peripheral Nervous System, sympathtic trunk</td>
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<tr>
<td>3</td>
<td>CETP</td>
<td>AAH25739</td>
<td>493 aa</td>
<td>Pancreas, Spleen, adult pooled</td>
</tr>
<tr>
<td>4</td>
<td>INSR</td>
<td>AAH47591</td>
<td>743 aa</td>
<td>Testis</td>
</tr>
<tr>
<td>5</td>
<td>LMNA</td>
<td>AAH33088</td>
<td>465 aa</td>
<td>Pancreas, adenocarcinoma</td>
</tr>
<tr>
<td>6</td>
<td>LPL</td>
<td>CAG33335</td>
<td>475 aa</td>
<td>No tissue type</td>
</tr>
<tr>
<td>7</td>
<td>NOS3</td>
<td>AAH69465</td>
<td>1203 aa</td>
<td>PCR rescued clones</td>
</tr>
<tr>
<td>8</td>
<td>PPARG</td>
<td>AAA80314</td>
<td>477 aa</td>
<td>bone marrow aspirate</td>
</tr>
<tr>
<td>9</td>
<td>TNF</td>
<td>BAE78639</td>
<td>233 aa</td>
<td>peripheral blood leukocyte</td>
</tr>
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</table>

Table 6.2. Showing the genes/proteins that have been studied in the present study, which are believed to be involved in Insulin Resistance Syndrome and Coronary artery disease.
<table>
<thead>
<tr>
<th>S.no</th>
<th>Gene Name</th>
<th>Ac. No</th>
<th>Length</th>
<th>Tissue Type</th>
</tr>
</thead>
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<td>ACE</td>
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<td>42 aa</td>
<td>No tissue type</td>
</tr>
<tr>
<td>2</td>
<td>ADIPOQ</td>
<td>AAH54496</td>
<td>244 aa</td>
<td>Peripheral Nervous System, sympathetic trunk</td>
</tr>
<tr>
<td>3</td>
<td>ADM</td>
<td>CAG46792</td>
<td>185 aa</td>
<td>No tissue type</td>
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<tr>
<td>4</td>
<td>AGER</td>
<td>AAH20669</td>
<td>404 aa</td>
<td>Lung</td>
</tr>
<tr>
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<td>AKR1B1</td>
<td>AAH05387</td>
<td>316 aa</td>
<td>Bladder, carcinoma</td>
</tr>
<tr>
<td>6</td>
<td>APOE</td>
<td>AAB59546</td>
<td>317 aa</td>
<td>Liver and blood</td>
</tr>
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<td>7</td>
<td>CD14</td>
<td>CAG33297</td>
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<td>CRP</td>
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<td>F7</td>
<td>CAI41382</td>
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<td>10</td>
<td>NOS</td>
<td>AAB32796</td>
<td>165 aa</td>
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<tr>
<td>11</td>
<td>TNF</td>
<td>CAI41940</td>
<td>233 aa</td>
<td>No tissue type</td>
</tr>
</tbody>
</table>

Table 6.3. Table showing the genes/proteins that have been studied in the present study, which are believed to be involved in diabetic associated with stroke.

<table>
<thead>
<tr>
<th>S.no</th>
<th>Gene Name</th>
<th>Ac. No</th>
<th>Length</th>
<th>Tissue Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACE</td>
<td>AAH36375</td>
<td>739 aa</td>
<td>Testis</td>
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<td>2</td>
<td>APOB</td>
<td>AAH51278</td>
<td>825 aa</td>
<td>Liver, hepatocellular carcinoma</td>
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<td>3</td>
<td>EPO</td>
<td>AAF23134</td>
<td>193 aa</td>
<td>No tissue type</td>
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<tr>
<td>4</td>
<td>FGB</td>
<td>AAH07030</td>
<td>173 aa</td>
<td>Liver</td>
</tr>
</tbody>
</table>

Table 6.4. Table showing the genes/proteins that have been studied in the present study, which are believed to be involved in Peripheral Vascular disease.

Figure 6.1. Phylogenetic tree that was constructed based on the alignment scores of all the protein sequences involved in diabetic cardiomyopathy.
Figure 6.2. Phylogenetic tree that was constructed based on the alignment scores of all the protein sequences involved in coronary artery disease.

Figure 6.3. The Phylogenetic tree that was constructed based on the alignment scores of all the protein sequences involved in diabetic associated with stroke.

Proteins with minimum distance
Figure 3. Schematic representation of the diabetic macrovascular complications with reference to bioinformatic and proteomic approaches for therapeutic drug target identification and/or biomarker identification.
References


