श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेंद्रम, तिरुवनन्तपुरम - 695 011, केरल, भारत
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
THIRUVANANTHAPURAM - 695 011, KERALA, INDIA
(एक राष्ट्रीय महत्व का संस्थान, विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार)
(An Institution of National Importance, Department of Science and Technology, Government of India)
टेलीफोन नं./Telephone No.: 0471-2443152 फैक्स/Fax: 0471-2446433, 2550728
ई-मेल/E-mail: sct@sctimst.ac.in वेबसाइट/Website: www.sctimst.ac.in

PROJECT COMPLETION REPORT

1. **Project number:** 5289

2. **Title of the project:** Mitochondrial metabolism and function in type 2 diabetic heart

3. **Funding Agency Name:** SERB, DST

4. **Project Reference Number provided by the Funding Agency:** NO.SB/SO/HS-051/2013

5. **Principal Investigator (Name & Address):** Dr. G. Srinivas, Scientist F, Department of Biochemistry, SCTIMST, Trivandrum, Kerala

6. **Co-Investigators (Name & Address):**

   i. Prof. Jayakumar K, Head, Dept. of CVTS, SCTIMST, Thiruvananthapuram, Kerala.
   ii. Dr. Vivek V. Pillai, Assistant Professor, Dept. of CVTS, SCTIMST, Thiruvananthapuram, Kerala.

7. **Implementing Institution:** SCTIMST

8. **Collaborating Institutions:** Nil

9. Date of Commencement: 18/06/2014

10. **Duration**: 3 years

11. **Date of completion**: 17/06/2018

12. **Objectives as approved**:

- **To analyze the glucose level/HbA1c, insulin level, C peptide and lipid profile in blood serum**

  It is necessary to identify the status of insulin and C peptide levels in the blood stream of both control and diabetic groups since it influences the metabolic status of cardiac tissue. This could be achieved by insulin and C peptide assay kits (ELISA), which are reliable, less time consuming and suitable for conducting large number of samples.
  The information on blood glucose level/HbA1c could be obtained from the clinical lab since it is done routinely in our hospital. Lipid profile of these patients can also be determined from our clinical lab.

- **To analyze the level of triglyceride accumulation in the collected tissue**

  Since hyperlipidemia is one of the features of diabetes, increase triglyceride accumulation is

expected to observe in diabetic heart. It is possible to isolate and determine the total triglyceride content by colorimetric assays. This assay helps to know the level of triglyceride that has accumulated and it could help to describe the altered lipid metabolism due to T2DM.

- **To study diabetic cardiac mitochondrial substrate utilization and ATP synthesis**

  This will be achieved by oxygraph system, where mitochondria will be isolated from cardiac tissue in suitable buffer and parameters like state 2, state 3, and state 4 respirations, ADP/O ratio, ATP synthase activity will be determined in the presence of different substrates. Oxygen consumption rate could be measured when substrates are added to the isolated mitochondria and then the data obtained could be compared for analysis.

  Also ATP levels will be analyzed in both diabetic and non-diabetic heart tissues

- **To determine the level of oxidative stress in cardiac tissue**.

  A marker for ROS generation is the GSH/GSSG ratio. A low ratio indicates high ROS level. Assay kits for quantification (based on the principle of luminescence or fluorescence) of their levels are available. These methods can give adequate information on tissue oxidative stress level.

- **Effects of reactive species on proteins as a measure of oxidative stress (protein oxidation, protein nitrotyrosine, protein S-nitrosylation etc.)**

  Levels of nitrotyrosine, S-nitrosylation and carbonyl groups in proteins from tissue lysates indicate oxidative stress in the tissues. These can be quantified by either separating the protein by SDS-PAGE and then immunoblotting or by Dot blot and then immunodetection.

13. **Deviation made from original objectives if any, while implementing the project and reasons thereof:** NA

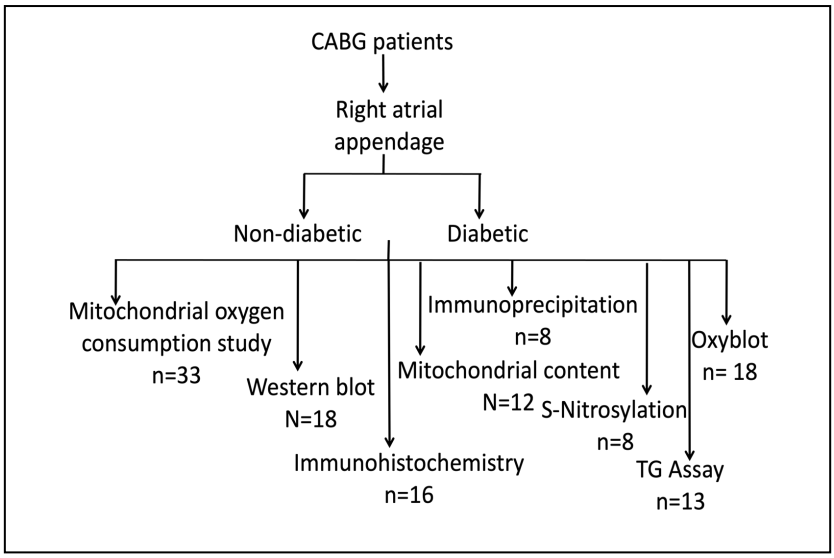
14. **Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs**:

14.1. Experimental set up and Methods

14.1.1. Human atrial appendage collection and processing

Human right atrial appendage was obtained while cannulation of right atrium during coronary artery bypass graft (CABG) surgery. A sample of the appendage was dissected and immediately rinsed in ice-cold buffer. For mitochondrial oxygen consumption studies, the sample was immersed in ice-cold BIOPS solution and immediately taken for mitochondrial isolation. Tissue samples for immunohistochemistry were immersed in buffered formalin solution. For all other studies the tissue samples were immediately stored at -80^{o}C.

Figure14.1.1. Flow chart representing the number of samples taken for each experiment



14.1.2. Patient Characteristics

The study was approved by the Institutional Ethics Committee (IEC) of Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum. Informed consent was obtained from patients admitted to the Department of Cardiovascular and Thoracic Surgery of SCTIMST undergoing CABG.All clinical data pertaining to the patients who participated in this study are shown in Table 1. The patients were grouped as either non-diabetic or diabetic by clinical diagnosis based on random blood glucose values and glycated hemoglobin (HbA1c) values of >6.2%. Patients with type 1 diabetes mellitus,atrial fibrillation, or left ventricular ejection fractions less than 30% were excluded from this study.

14.1.3. Experimental animal model

C57BL/6 male mice, 8 weeks of age, were used for the study. The mice were mainly grouped in two, 1) control 2) diabetic. T2DM was developed in mice by intraperitoneal injection of two doses of 120mg/kg bodyweight streptozotocin (STZ) with 100mg/kg body weight nicotinamide (NA) each at alternate days after a 16 h fasting period. The control animals were injected with vehicle control, citrate buffer, pH4.5. Development of T2DM was analyzed by evaluating blood glucose levels at different time points, 14 days after the STZ injection. For that, a drop of blood was obtained from the tail tip and glucose levels were measured using glucometer and glucose strips. Also their HbA1c levels were checked just before euthanasia by cervical dislocation. Weekly analysis of glucose levels and body weight were performed and recorded. The diabetic mice were again divided to two groups. One group of mice are maintained hyperglycemic for 2 weeks and then euthanized for heart tissue and the other maintained hyperglycemic for 10 weeks and euthanized.

14.1.3.1. Oral Glucose Tolerance Test (OGTT)

The mice were subjected to 12h fasting and then fed with glucose solution (2mg/total body weight) in 200µl water. Glucose values were measured from blood obtained from tail tip incision before and after (at 10, 20, 30, 60 and 120 min) ingestion of glucose.

14.1.4. Mitochondrial isolation

Immediately after collecting the atrial biopsy from humans/ whole heart from mice the tissues were immersed in ice-cold BIOPS buffer and were transported to the laboratory for mitochondrial isolation. Mitochondria were isolated from tissue by differential centrifugation, as described by Fontana et al., published in Mitochondrial Physiology Network OroborosO2k protocols (2015). Mitochondria were immediately used for oxygen consumption measurements.

14.1.5. Mitochondrial oxygen consumption rate

A 10 µl aliquot of the mitochondrial suspension was added to each 2-ml Oxygraph O2k chamber containing mitochondrial respiration buffer (MiRO5). Data acquisition and analysis of oxygen consumption rate (OCR) were performed with Datlab5 software (Oroboros, Innsbruck, Austria). Substrate, uncouplersor inhibitors for respiratory experiments were added in the respiration medium in a step-by-step manner with the use of micro syringes.

Two mitochondrial substrate- uncoupler- inhibitor titration protocols (SUIT) were applied: fatty acid +carbohydrate protocol (chamber A) and carbohydrate protocol (chamber B). In the fatty acid + carbohydrate protocol, state 2 respiration was measured with palmitoyl- L-carnitine (Pal) (25 µmol/L) and malate (M) (2mmol/L) ($PalM_0$), followed by the addition of ADP (5 mmol/L) feeding electrons into electron transferring flavoprotein (ETF) and complex I (state3 respiration) ($PalM_{ADP}$) followed by the carbohydrate protocol, where pyruvate, glutamate and succinate are sequentially added. In the carbohydrate protocol, substrate combinations were used for electron flow through complexes I and II. Complex I–dependent state 2 respiration was determined in the presence of 10 mmol/L glutamate (G) and 2mmol/L malate (GM_0). Complex I–dependent state 3 respiration (GM_{ADP}) was determined as a phosphorylation-stimulated respiration rate in the presence of 5mmol/L ADP. In all groups, the lack of a significant increase in respiration after the addition of cytochrome c (2 µmol/L) confirmed the integrity of the outer mitochondrial membrane. Subsequently, pyruvate (P) stimulated complex I dependent state 3 respiration (GMP_{ADP}) was determined followed by succinate (S) stimulated complex II respiration ($GMPS_{ADP}$). Complex I was inhibited by rotenone (0.05 µmol/L), and complex II- dependent state 3 respiration (S_{Rot}) was determined in the presence of 10 mmol/L succinate. State 4 respiration (S_{Omy}) was determined by addition of oligomycin (2.5 µmol/L). Complex II- dependent uncoupled state of respiration (S_{FCCP}) was determined in presence uncoupler FCCP (1 µmol/L). Finally, antimycin-A (2.5 µmol/L) was added to inhibit complex III- mediated respiration.

14.1.6. Chemicals for mitochondrial SUIT protocols

14.1.6.1. Substrates

Substrate	FW	Stock solution Conc.(mM)	Stock solution Amount	Final concentration in 2ml	
				Chamber A	Chamber B
G: L- Glutamic acid	1614.1	2000	3.382g/ 10ml H_2O	10mM	10mM
M: L-Malic acid	134.1	400	536mg/10ml H_2O	2mM	2mM
P: Pyruvic acid sodium salt	110	2000	44mg/ 0.2ml H_2O	5mM	5mM
S: Succinate disodium salt, hexahydrate	270.1	1000	2.701g/ 10ml H_2O	10mM	10 mM
Pal: Palmitoyl-DL-carnitine-HCl	436.1	10	8.72 mg/ 2ml H_2O	25 µM	-
c: Cytochrome c	12500	4	50mg/ ml H_2O	2µM	2µM
D: ADP (Adenosine 5' diphosphate, potassium salt)	501.3	500	0.501g/ 2ml H_2O	5 mM	5 mM

14.1.6.2. Uncouplers

Uncouplers	FW	Stock solution Conc (mM)	Stock solution Amount	Final concentration in 2ml	
				Chamber A	Chamber B
F(FCCP): Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone	254.2	1	2.54mg/ 10ml ethanol	0.5+0.5 µM	0.5+0.5 µM

14.1.6.3. Inhibitors

Inhibitors	FW	Stock solution Conc.(mM)	Stock solution Amount	Final concentration in 2ml	
				Chamber A	Chamber B

AA: Antimycin A	540	5	11mg/ 4ml ethanol	2.5µM	2.5µM
Omy: Oligomycin	800	5	4mg/ 1ml ethanol	2µM	2 µM
Rot: Rotenone	3144.4	1	3.144mg/10ml ethanol	0.1µM	0.1µM

14.1.7. Western blotting

Cardiac tissue wascut into very small pieces using scissors and transferred to an ice-cold homogenizer. The tissue pieces were ground thoroughly in ice-cold Radio ImmunoPrecipitation Assay (RIPA) buffer containing proteinase inhibitor cocktail and then the lysate was transferred to a microcentrifuge tube. The lysate was vortexed intermittently for 1 h(every 5 min). Tissue lysates were centrifuged at 13,000 rpm for 20 min and the supernatants were stored at -80^{0}C. Extracted proteins were quantified using Pierce 660nm method (Pierce Biotechnology, Massachusetts, USA). Extracted proteins (100 µg protein) were heat denatured for 5 min and resolved on Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) at 100V. Resolved proteins were transferred on to nitrocellulose membrane at 10V for40min and the membrane was blocked for 1 h at room temperature with blocking buffer (1% BSA in TBST). The membrane was then probed with antibodies specific proteins at 4^{o}C overnight. HorseRadish Peroxidase (HRP) conjugated secondary antibodies (anti rabbit IgG; 1:1000, anti mouse IgG; 1:100000) were used. Bands were visualized using West Pico/ West FemtoChemiluminescence Detection Kit (Pierce Biotechnology, Massachusetts, USA) or OptiBlot (Abcam, Cambridge, UK). Equal volumes of luminol and peroxide solutions were mixed and added on to the membranes. Light emitting bands were captured on an X-ray film and developed and then documented in Gel Doc™ XR Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using Quantity One 1 D Analysis Software.

14.1.8. Mitochondrial DNA copy number

The Mitochondrial DNA copy number was determined by real-time qPCR with SYBR detection, using primers specific for the $tRNA_{Leu}$(UUR) gene (Forward 5'C ACCCAAGAACAGGGTTTGT3') (Reverse 5'TGGCCATGGGTATGTTGTTA3') of the mitochondrial genome and the nuclear β-2-microglobulin (β2M) (Forward 5'TGCTGTCTCCATGTTTGATGTATCT3')
(Reverse5'TCTCTGCTCCCCACCTCTAAGT3') gene as previously described (Baiand Wong, 2005; Dimmock et al., 2010). The intensities of fluorescent signals produced by SYBR chemistry are then analyzed by a suitable detection system.

14.1.14. Intramyocardial triglyceride measurement

Triglycerides were measured in atrial muscle homogenate using a colorimetric assay kit procured from BioVision, California, USA, according to the manufacturer's instructions. Briefly, the tissue samples were cut to small pieces and then ground in a homogenizer with 5%

NP-40. The lysate was then heated at 140ºC for 2 min or till the solution turns cloudy. The lysate is cooled and again heated for 2 min and a brief spin of lysate in a microcentrifuge gave a clear supernatant that contained tissue triglycerides. The samples then subjected to enzymatic lysis using the enzyme mix. A pink color reaction takes place after incubating the mix for 20 min in dark and the absorbance is read at 510nm. Triglyceride concentration in samples was calculated by the following formula:

Triglyceride concentration = (Amount of triglyceride obtained from standard curve nmol/ volume of sample added µl) x sample dilution factor

14.1.10. Immunoprecipitation

Tissue samples were lysed in NP40 lysis buffer. After incubating the tissue lysate (500µg) with 2µg of primary antibody at 4^0C for 1h, protein A/G beads (Pierce Biotechnology, Massachusetts, USA) were added. This mixture was incubated overnight at 4^0C followed by sequential steps of washing and centrifugation for removing unbound proteins. The bound proteins were eluted by boiling the beads at 145^0C for 5 min in laemmli buffer followed by centrifugation at 14,000rpm.

14.1.11. Immunohistochemistry

Biotin- free immunoenzymatic antigen detection system (Abcam, Cambridge, UK) was employed for immunohistochemical studies (3-nitrotyrosine detection). Briefly, 5 µm-thick tissue sections were cut from buffered formalin-fixed, paraffin-embedded atrial biopsies. Sections were deparaffinised and rehydrated. Antibodies to 3-nitrotyrosine were used at a dilution of 1:100 and incubated overnight at 4ºC. Immunohistochemical staining was done by peroxide diamnobenzidine method (DAB) and then counterstained with hematoxylin. The sections are then dehydrated and stabilized with mounting medium. Images of tissue sections were taken in a microscope at 40X and quantified using Image J software.

14.1.12. Statistical Analysis

All values are given as means $\pm$ SEM. A p- value of less than 0.05 was considered as significant for all statistical evaluations. Comparisons between two groups were performed by Student's t test where ever the underlined distribution is normal otherwise Mann Whitney U test was used. SPSS tool was used for statistical evaluations and graphs were prepared by Graph pad Prism.

15. Detailed analysis of Results

15.1.1. Patient Characteristics

Atrial biopsies were obtained from 126 human subjects, during a period of 24 months and those tissue samples were used for mitochondrial functional analysis and other experiments. The characteristics of the patients are presented in Table 15.2.1.

Table 15.1.1.Patient characteristics

Characteristics	**Non diabetic (n=514)**	**Diabetic (n=67)**
Age, years	56.14± 1.4	514.38± 1.1
Male, n (%)	53 (814.8)	64 (145.5)
BMI	23.42± 0.75	25.27± 0.414*
HbA1c, %	6.21± 0.12	7.87± 0.114***
Random blood glucose, mg/dL	104.15± 5.01	1614.67± 7.86***
Hypertension, n (%)	114 (32.2)	58 (86.6)
LVEF	62.12± 1.41	62.03± 1.2
Total cholesterol, mg/dL	161.4± 7.7	142.04± 6.06
HDL cholesterol, mg/dL	36.146± 2	36.74± 1.16
Triglycerides, mg/dL	41.44± 6.7	30.66± 4.4
NYHA class	class II	class II
Preoperative medications ***(data of patients included in mitochondrial respiration studies only: non-diabetic n=11, diabetic n=22)***	***n=11***	***n=22***
Insulin, n (%)		
Metformin, n (%)	-	17 (77)
Sulfonylureas, n (%)	-	8 (36)
β-blockers, n (%)	-	5 (22.7)
Statins, n (%)	10 (140.14)	12 (54.5)
Diuretics, n (%)	14 (40.14)	14 (40.14)
Antiplatelets, n (%)	7 (31.8)	14 (40.14)
ACE inhibitors or ARBs, n (%)	7 (31.8)	7 (31.8)
Calcium channel blocker, n (%)	3 (27.3)	5 (22.7)
	-	4 (18.2)

*The total number of non-diabetic patients selected for various experiments in the study is 514 and that of diabetic patients is 67. The HbA1c and random blood glucose levels vary significantly between the 2 groups, while all other parameters are matched, except BMI, which was also varied significantly in diabetic patients when compared with non-diabetic patients. (p-value *<0.05, ***<0.001)*

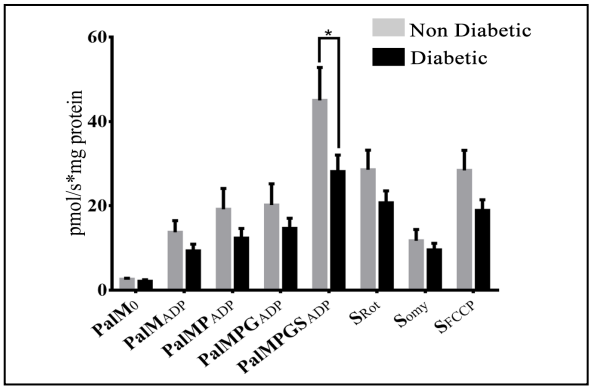
The patient subjects we have selected were grouped to non-diabetic and diabetic based on their HbA1c and random blood glucose levels and there were 514 (46.8%) non-diabetic, and 67 (53.17%) diabetic subjects. The diabetic group was having a mean HbA1c level of 7.87% and mean random blood glucose level of 1614.67mg/dL that were significantly higher than non-diabetic group. All other parameters were not significantly different including age, triglyceride level, cholesterol level, left ventricular ejection fraction and NYHA class.

15.2.2. Absence of fatty acid- mediated cardiac mitochondrial dysfunction in diabetic patients

To determine whether T2DM alter cardiac mitochondrial function, we checked the

mitochondrial respiration in isolated mitochondria by high-resolution respirometry. After the addition of 10µl aliquot of the mitochondrial suspension to each of the chambers, the sequential addition of palmitoyl L- carnitine, malate, ADP, pyruvate, glutamate, succinate, rotenone, oligomycin, FCCP, and antimycin showed no significant difference in the state 2 and state 3- complex I respiration in diabetic mitochondria, but significant lower oxygen consumption rate (OCR) in state 3- complex I +II mitochondria of diabetic subjects even though complex II alone (after addition of rotenone) showed no significant change in its respiration. Although palmitoyl L- carnitine and glutamate- mediated respiration were not significantly different, there is a decreasing trend observed in diabetic mitochondria (Figure 14.2.1). The OCR was expressed in pmol/s*mg of mitochondrial protein and the values are ROX (residual oxygen consumption rate) corrected. ROX represented the residual oxygen flux when electron transport due to some oxidative side reactions is inhibited in the mitochondria by antimycin and the flux values are normalized by ROX.

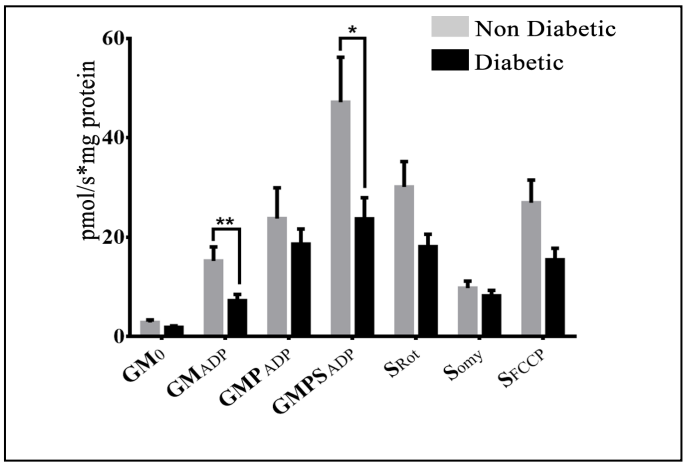
Figure 15.2.1. Diabetic mitochondria show unaltered fatty acid utilization



States of respiration of non- diabetic and diabetic mitochondria with substrates palmitoyl L-carnitine followed by pyruvate, glutamate and succinate, inhibitors rotenone and oligomycin and the uncoupler FCCP. $PalM_0$- State 2 respiration, $PalM_{ADP}$- State 3 respiration of palmitoyl L- carnitine, PalMP- State 3 respiration of both palmitoyl L-carnitine and pyruvate, PalMPG- State 3 respiration of palmitoyl L-carnitine with pyruvate and glutamate, PalMPGS- State 3 respiration of palmitoyl L-carnitine with pyruvate, glutamate and succinate, S_{Rot} – State 3 respiration of succinate after inhibition of complex I activity by rotenone, S_{Omy}- State 4 respiration byoligomycin, where complex V activity is blocked. Values are represented as means ± SEM. (p-value <0.05)*

In other chamber, the sequential addition of glutamate and malate, ADP, pyruvate, succinate, rotenone, oligomycin, FCCP, and antimycin revealed no significant difference in the state 2 OCR in diabetic mitochondria, but significant lower state 3-complex I and state 3-complex I +II respiration in diabetic subjects eventhough complex II alone (after addition of rotenone) showed no significant change in its respiration (Figure 15.2.2). The OCR was expressed in pmol/s*mg of mitochondrial protein. However, state 3 respiration supported by pyruvate in addition to glutamate/ malate, eventhough showed a decreasing trend, was not statistically significant in the diabetic mitochondria.

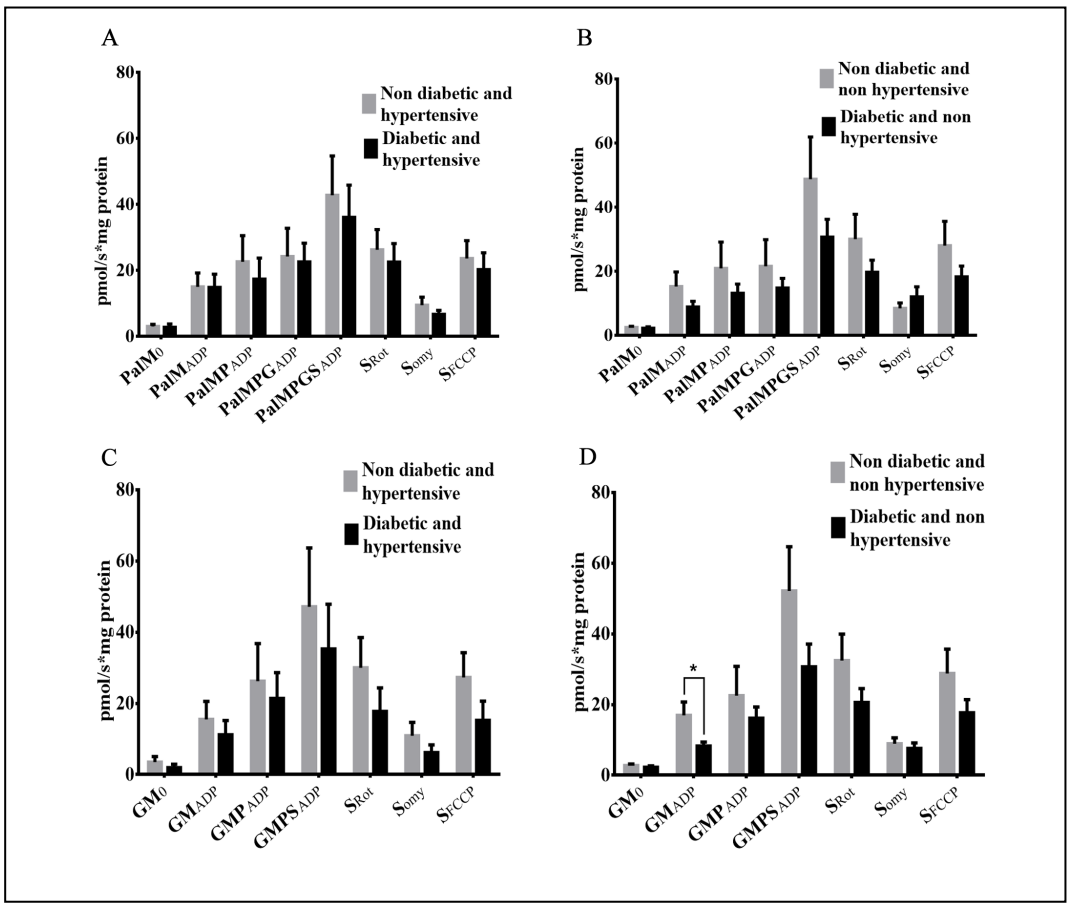
Figure 15.2.2. Diabetic mitochondria show altered glutamate-mediated complex I respiration



States of respiration of non- diabetic and diabetic mitochondria with substrates glutamate, pyruvate and succinate, inhibitors rotenone and oligomycin and the uncoupler FCCP.GM_0- State 2 respiration, GM_{ADP}- glutamate-mediated state 3 respiration of complex I, GMP- glutamate and pyruvate-mediated state 3 respiration of complex I, GMPS- glutamate/pyruvate-mediated state3 respiration of complex I and succinate- mediated state 3 respiration of complex II, S_{Rot} – succinate-mediated state 3 respiration of complex II after inhibition of complex I by rotenone, S_{Omy}- State 4 respiration byoligomycin, where complex V activity is blocked.Values are represented as means $\pm$ SEM. (p-value <0.05, **<0.01)*

Since there were both hypertensive and non-hypertensive patients included in this study, we further classified them based on hypertension within the non-diabetic and diabetic groups.It was observed that irrespective of the status of hypertension, the fatty acid substrate utilization was not changed significantly in diabetic mitochondria (Figure 15.2.3 (A),and (B)). It was noticed that state 3 glutamate-mediated complex I respiration was decreased significantly in non-hypertensive diabetic mitochondria than the hypertensive diabetic mitochondria (Figure 15.2.3 (C) and (D)).

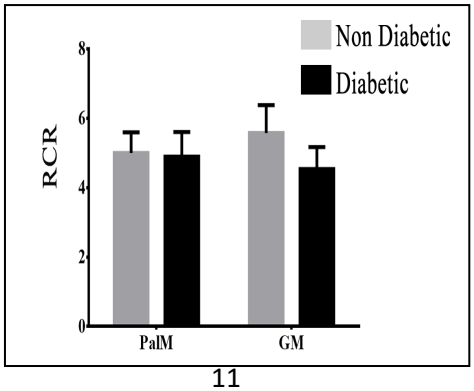
Figure 15.2.3: Fatty acid substrate utilization was unchanged irrespective of the status of hypertension



(A) Bar graph showing states of respiration of mitochondria of hypertensive- non-diabetic and diabetic patients with fatty acid + carbohydrate protocol. (B) Bar graph showing states of respiration of isolated mitochondria of non-hypertensive - non-diabetic and diabetic patients with fatty acid + carbohydrate protocol. (C) Bar graph showing states of respiration of mitochondria of hypertensive - non-diabetic and diabetic patients with carbohydrate protocol. (D) Bar graph showing states of respiration of isolated mitochondria of non-hypertensive – non-diabetic and diabetic patients with carbohydrate protocol. Error bars represent ± SEM.

The decrease in glutamate-mediated respiration was not evident when respiratory control ratio (RCR) was calculated (RCR is the ratio between oxygen flux in ADP stimulated state/ leak) (Figure 15.2.4).

Figure 15.2.4. Respiratory control ratio



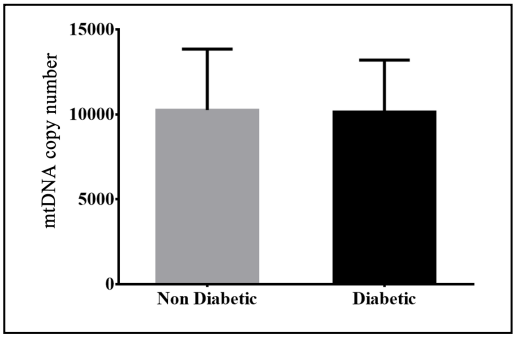
Palmitoyl L-carnitine- and glutamate-mediated respiratory control ratio is shown to be similar in both non-diabetic and diabetic mitochondria. Values are represented as mean ± SEM.

Findings:

- **Absence of altered fatty acid-mediated state3 complex I respiration**
- **Altered glutamate-mediated state 3 complex I and succinate-mediated state3 complex II respiration in diabetic group**
- **Unaltered fatty acid-mediated respiration in both hypertensive and non-hypertensive diabetic group, while decreased glutamate-mediated respiration in diabetic-non hypertensive group**

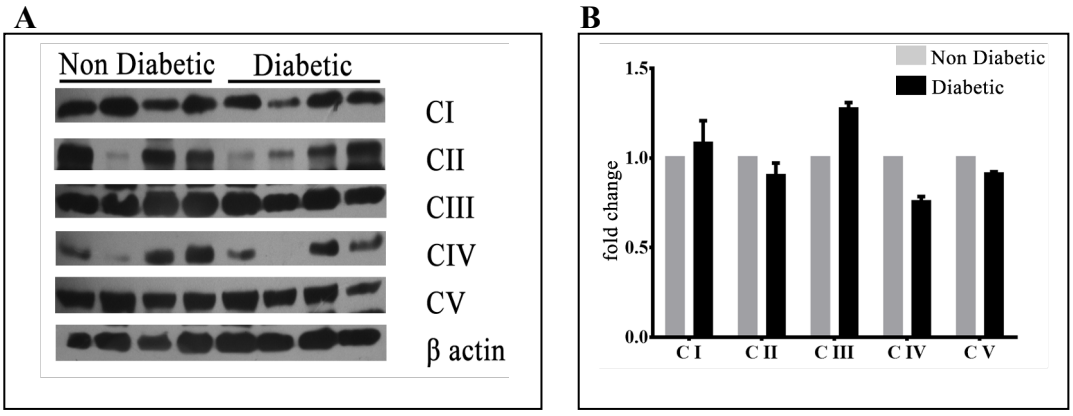
The mitochondrial copy number was also unchanged in diabetic subjects compared to the non-diabetic group of patients (Figure 15.2.5). In summary, diabetes status was not associated with fatty acid- mediated mitochondrial respiration rates. We then explored electron transfer chain complex levels in atrial tissue homogenates. None of the complex (I-V) levels vary much among the non-diabetics and diabetics (Figure 15.2.6).

Figure 14.2.5. Mitochondrial DNA copy number



No significant change in the mitochondrial content in diabetic cardiac tissue is shown by qRT PCR analysis.Values are represented as mean ± SEM.

Figure 15.2.6. Expression of oxidative phosphorylation (OXPHOS) complexes



(A) Mitochondrial OXPHOS complex expression is unchanged in diabetic mitochondria as shown by western blot analysis. (B) Bar graph showing fold change in expression levels of

OXPHOS complexes in diabetic cardiac tissue with respect to non-diabetic cardiac tissue. Error bars represent ±SEM.

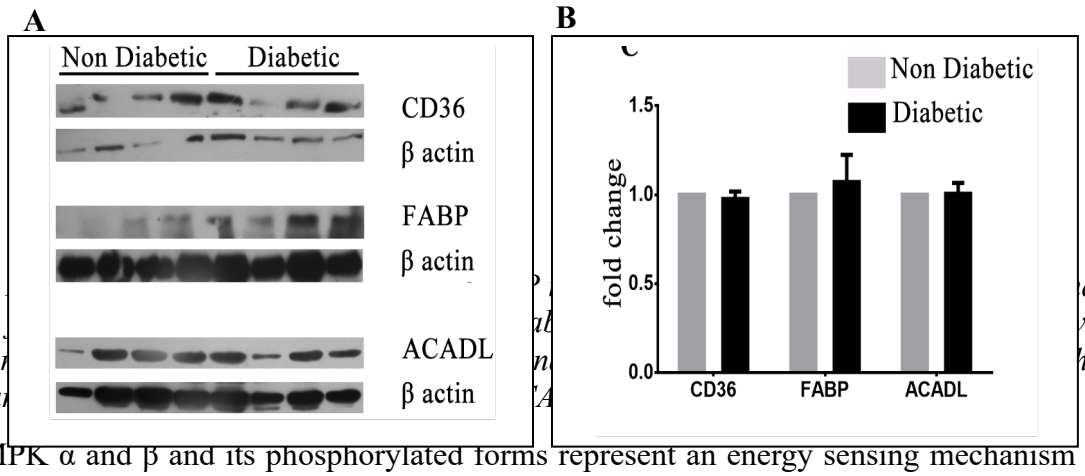
Findings:

- **Unaltered levels of mitochondrial content and expression of OXPHOS complexes**

15.2.3. Balanced fatty acid uptake and metabolism is seen in diabetic subjects

In order to check whether there is a significant difference in the fatty acid metabolism in diabetic subjects, we evaluated proteins responsible for fatty acid uptake, FABP and CD36. Levels of ACADL, an enzyme responsible for the metabolism of long chain fatty acid was also determined between the two groups. None of these proteins were significantly different among these groups (Figure 15.2.7).

Figure14.2.7. Expression of fatty acid uptake proteins and fatty acid oxidation enzyme



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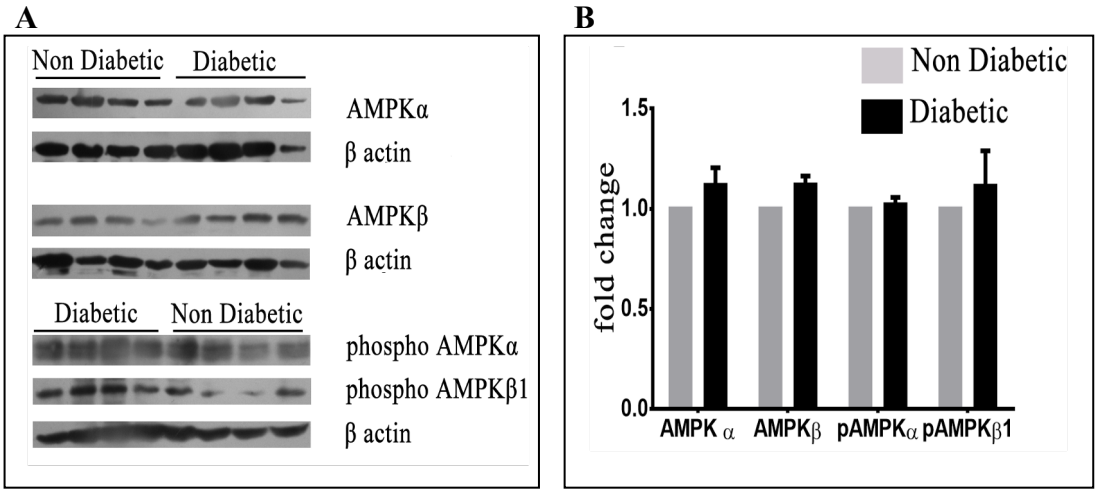
AMPK α and β and its phosphorylated forms represent an energy sensing mechanism in the cells and the phosphorylated form is responsible for inhibiting the activity of the enzyme acetyl-coA carboxylase (ACC), which thereby activates fatty acid utilization in the cells. Western blot analysis revealed that there is no significant difference in expression of AMPK subunits in diabetic human subjects (Figure 15.2.8). There appears to be no inhibition of β oxidation between the two groups.

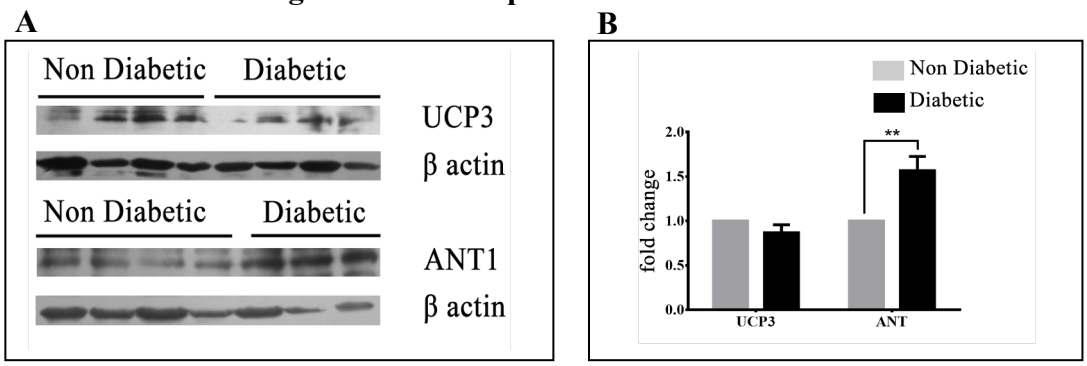
Figure15.2.8.Expressionof AMPK subunits



(A) Unaltered expression of AMPK subunits, AMPKα and AMPKβ and their phosphorylation status is showed by western blotting. (B) The bar graphs represent the fold change in expression of the respective subunits. Error bars represent ±SEM.

Also there was no change in expression for UCP3, while a higher expression of ANT1 in diabetic group was observed (Figure 14.2.14). Eventhoughincreased ANT1 expression levels indicated possible changes in ADP/ ATP exchange, the information on the activity of ANT1 is required inorder to confirm that the change in expression is also reflected in its activity. However, AMPK, UCP3 and ANT1 expression levels together suggested possible absence of deranged energy production in diabetic cardiac tissue.

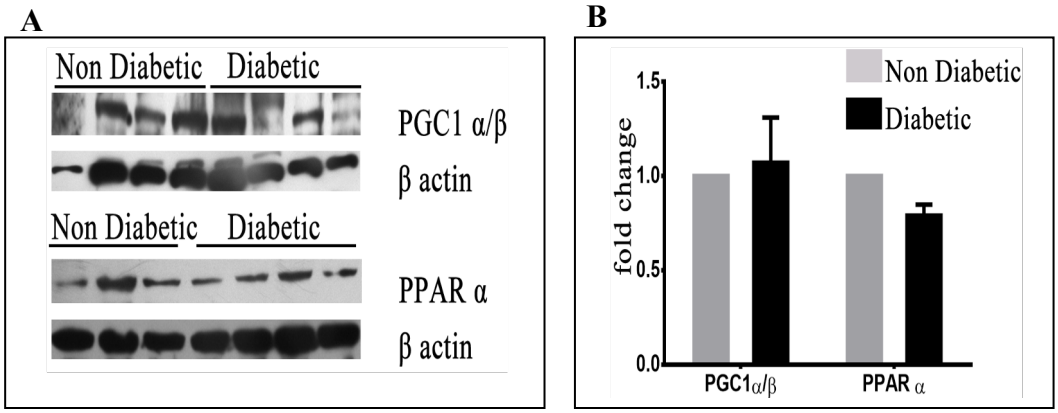
Figure 15.2.14. Expression of UCP3 and ANT1



(A)Unaltered expression of UCP3and increased levels of ANT1 in diabetic cardiac tissue is showed by western blotting. (B)The bar graphs represent the fold change in expression of the respective proteins. Error bars represent ±SEM.

PPAR α and PGC1-α/β, the nuclear binding proteins that promotes expression of genes for fatty acid uptake, β oxidation and for storage, also did not show any difference in expression (Figure 15.2.10).

Figure 15.2.10. Expression of PGC1α/β and PPARα



(A) Balanced levels of expression of PGC1α/β and PPARα are showed by western blotting. (B) The bar graphs represent the fold change in expression of the respective proteins. Error bars represent ±SEM.

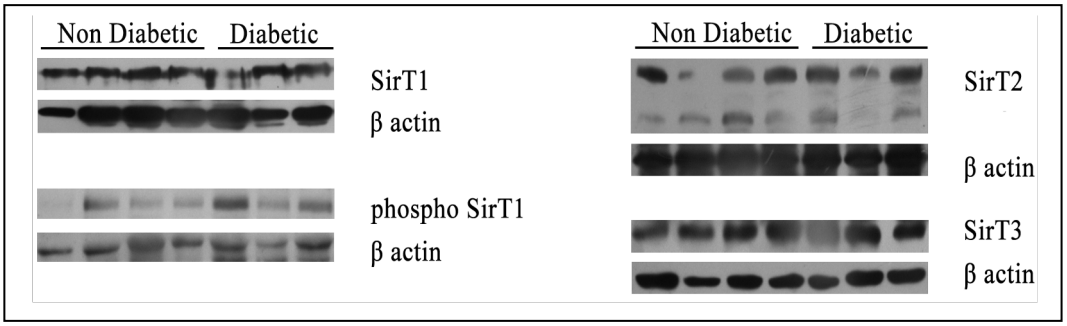
Findings:

- **Unaltered levels of fatty acid uptake proteinand fatty acid oxidation enzyme**
- **Absence of increased or decreased AMPK activation**
- **Balanced expression levels of UCP3, PGC1α/β and PPARα**
- **Increased ANT1 levels in diabetic group**

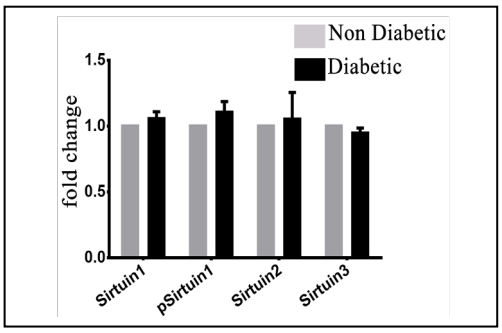
Expression of sirtuins, a group of NAD^+ dependent deacetylases that regulate gene expression or enzymes responsible for fatty acid metabolism via deacetylation were shown unchanged among the two groups (Figure 14.2.11). Also there was no significant difference in the whole cell and mitochondrial acetylated lysine levels (Figure 15.2.12) and the acetylation status of ACADL and ANT1 were unchanged (Figure 15.2.13). In summary, diabetes status was not associated with increased fatty acid uptake, metabolism or its regulators.

Figure 15.2.11. Expression of Sirtuins

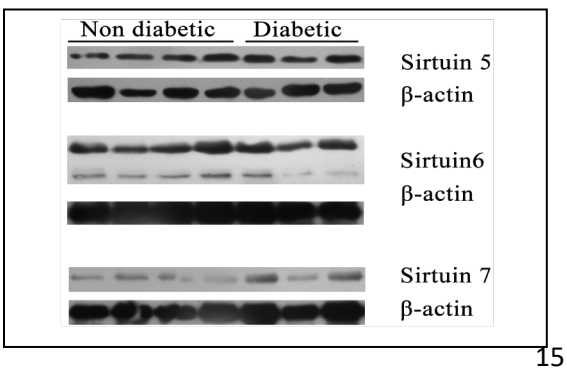
A



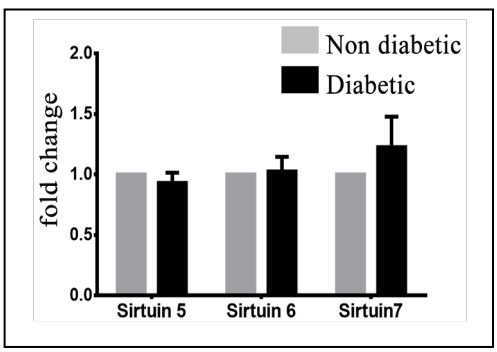
B



C

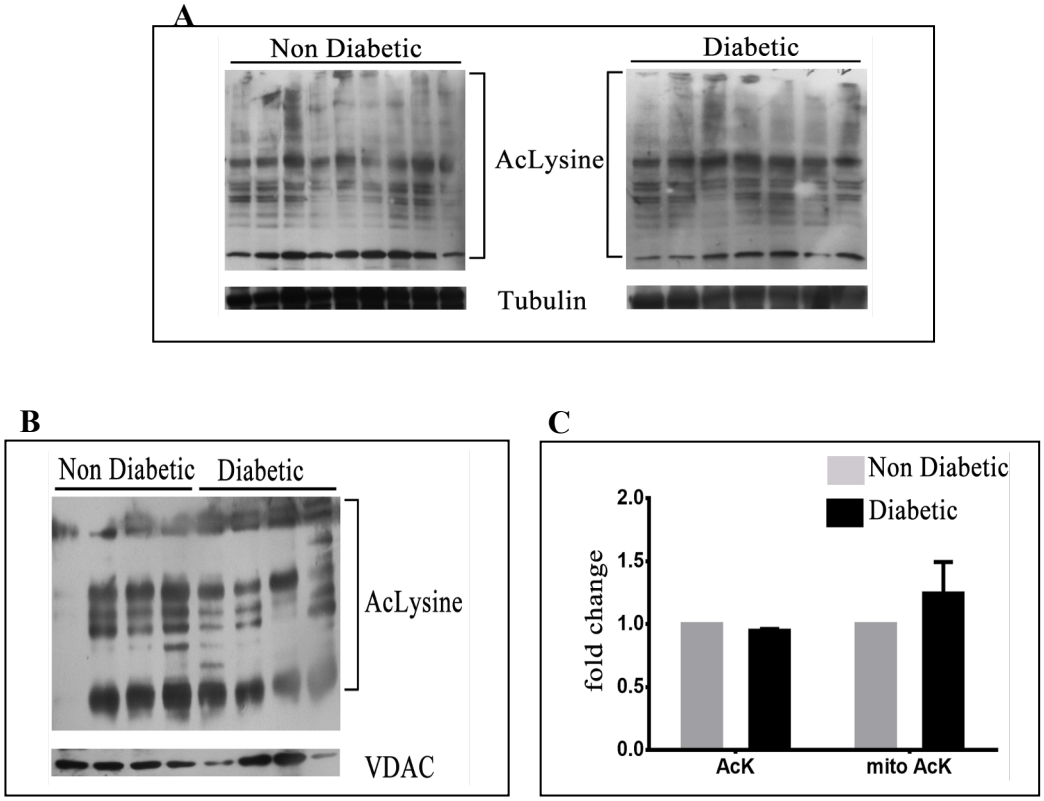


D



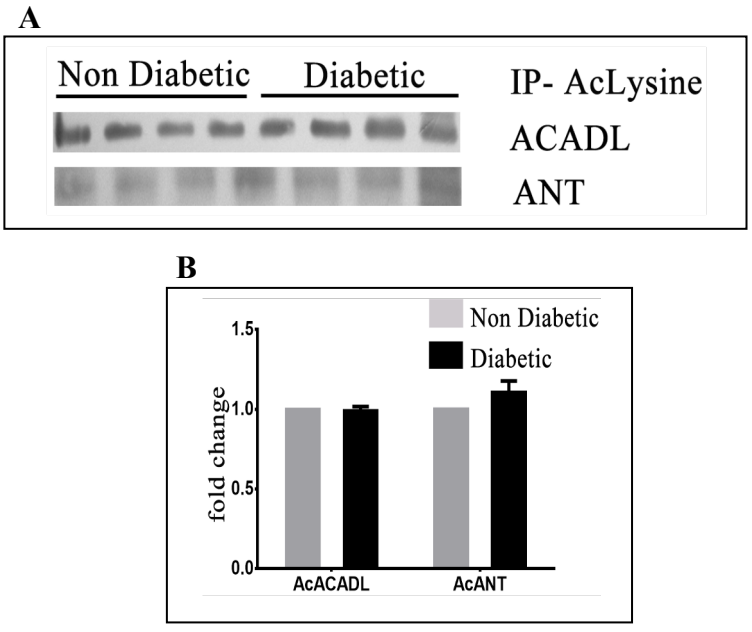
(A) The major nuclear sirtuin, sirtuin1,the cytoplasmic sirtuin, sirtuin 2 and the mitochondrial deacetylase, sirtuin 3 are unaltered in their expression. (C)Expression of other sirtuins that are having relatively less deacetylase activity, are also shown to be unchanged (sirtuin5, 6 and 7). (B and D) The bar graphs represent the fold change in expression of respective deacetylases.Error bars represent ± SEM.

Figure 15.2.12: Acetylated proteome



(A) Total acetylated proteome in diabetic cardiac tissue was not altered when compared with that of non-diabetic cardiac tissue. (B) Insignificant change in the level of mitochondrial acetylome was also observed in both groups. (C) The bar graph represents levels of total and mitochondrial acetylome. Error bars represent ± SEM.

Figure 15.2.13: Acetylation of ACADL and ANT1



(A) Acetylation of lysine residues in ACADL and ANT1 of diabetic cardiac tissue lysate was shown to be the same as that of non diabetic. (B) Bar graph represents fold change in levels of acetylation in respective proteins of diabetic patients with that of non-diabetic patients.

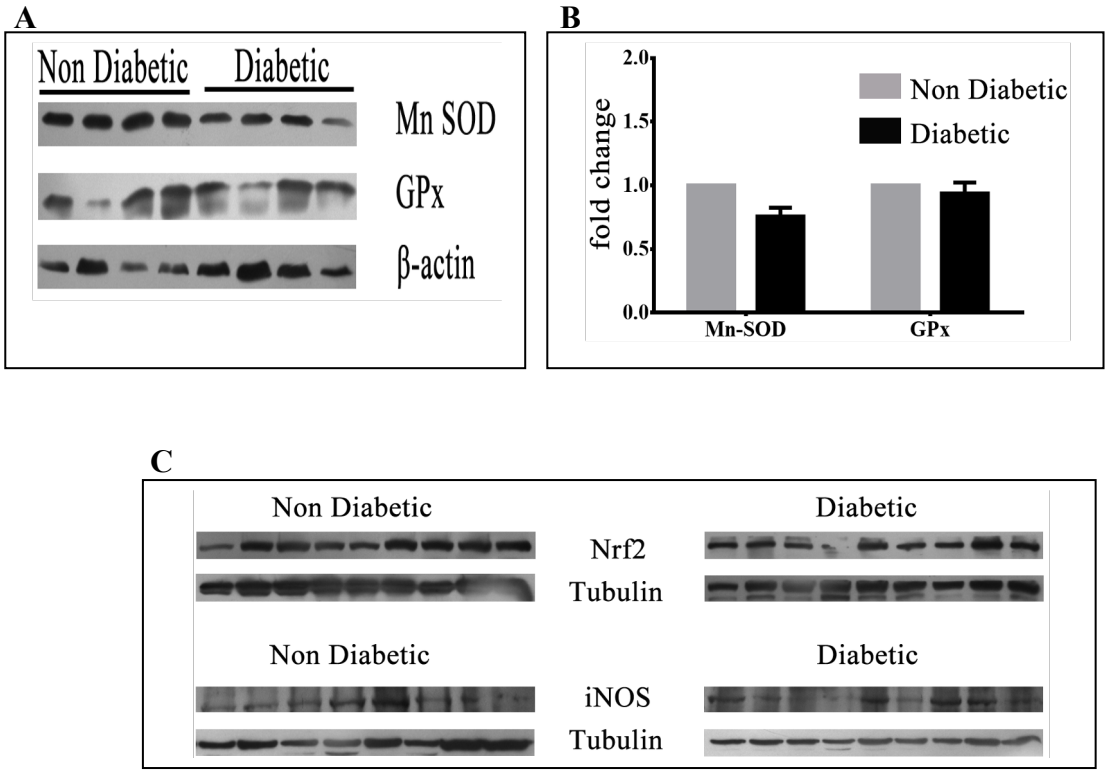
Findings:

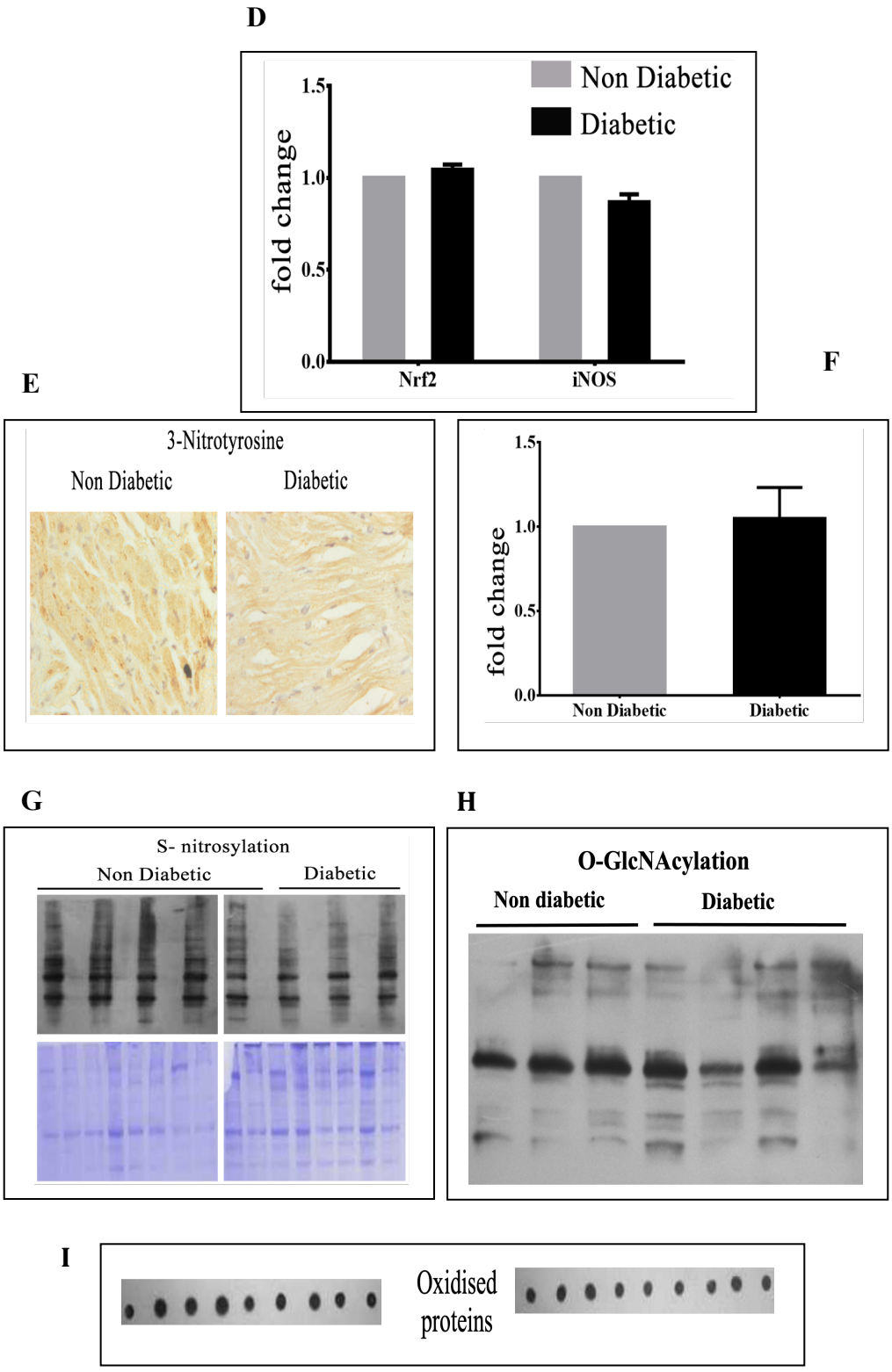
- **Unchanged sirtuin levels**
- **Absence of changes in total and mitochondrial acetylated protein levels**
- **Balanced acetylation status of lysine residues in specific proteins ACADL and ANT1**

15.2.4. Equivocal evidence for cardiac oxidative stress in diabetic patients

Considering the important role of mitochondria in oxidative stress, we next assessed ROS production and antioxidant systems in atrial tissues. Diabetic patients showed no increased myocardial oxidative stress, along with balanced expression of the antioxidant enzymes, that is, mitochondrion-located superoxide dismutase and cytosolic glutathione peroxidase (Figure 15.2.14(A and B)). In addition, Nrf2, which is antioxidant response protein, was equally present under diabetic conditions as in non-diabetic cases (Figure 15.2.14(C and D)). Inflammatory enzyme like iNOS (Figure 15.2.14(C and D)) and the posttranslational modifications, 3-nitrotyrosine (Figure 15.2.14(E)), S- nitrosylation (Figure 15.2.14(G)), OGlcNAcylation (Figure 15.2.14(H)) and carbonylation (Figure 15.2.14(I)) in the atrial tissue homogenates were also probed. Diabetes did not influence antioxidant enzymes or oxidative stress in atrial tissues.

Figure 14.2.14: Oxidative stress markers





Unchanged levels of Mn-SOD, GPx (A,B) and Nrf2 (C, D) in T2DM cardiac tissue showed absence of overt oxidative stress. One of the stress proteins, iNOSwas also shown to be expressed in unaltered levels in the diabetic cardiac tissue(C, D). The posttranslational modifications like 3-nitrotyrosine (E, F), S-nitrosylation (G), O-GlcNAcylation (H) and

carbonylation (I) which are indicators of oxidative stress, were also observed in unchanged levels.

Findings:

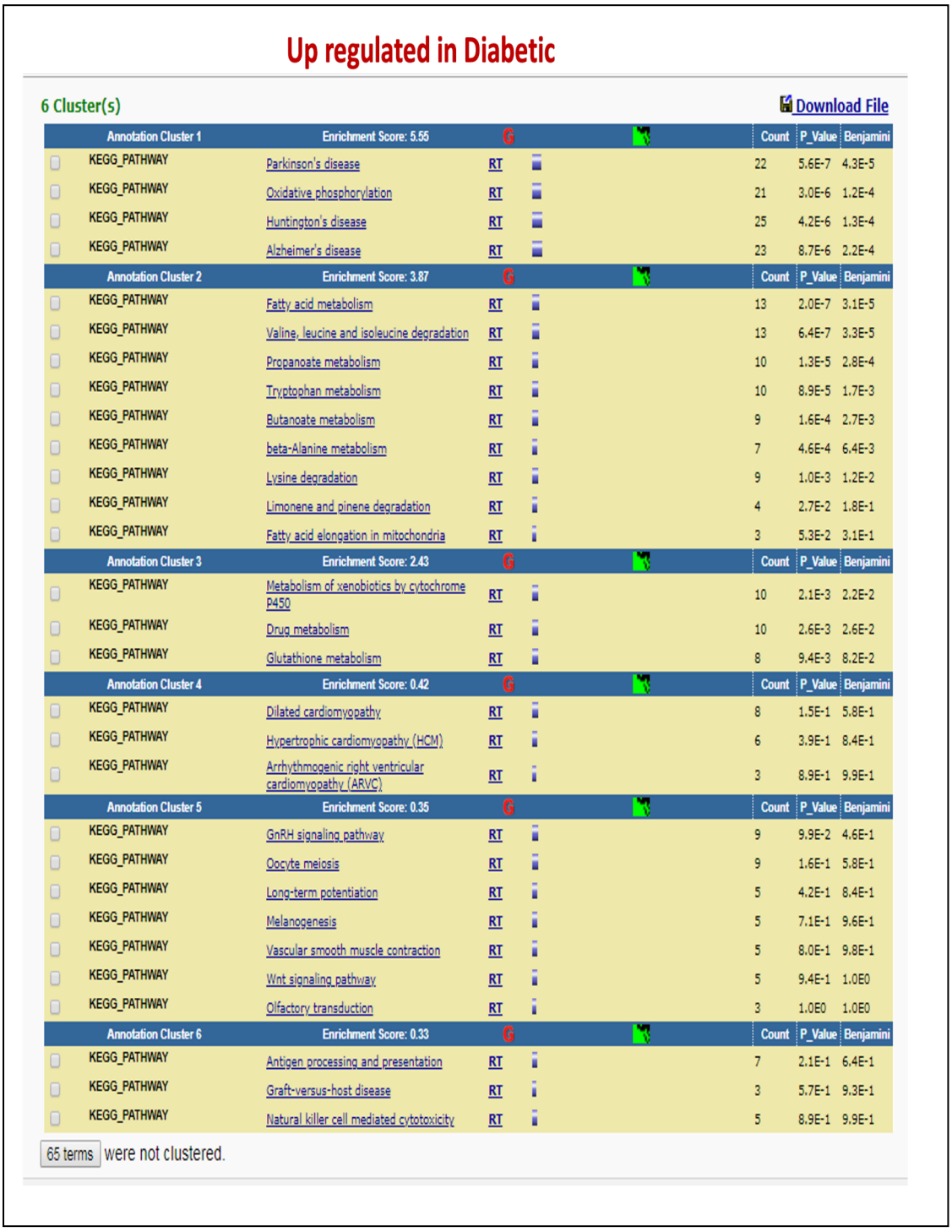
- **Unaltered levels of antioxidant enzymes in non-diabetic and diabetic groups**
- **Unchanged levels of oxidative stress markers**

15.2.5. Mass spectrometric analysis

Through mass spectrometry, the relative quantities of various proteins in diabetic human heart tissue were analysed and based on this data a pathway analysis was done where different cellular pathways were identified to be upregulated or downregulated under diabetic conditions. It was observed that under diabetic condition, some proteins in a pathway were upregulated and some others in that same pathway were downregulated (Figure 15.2.15 (A) and (B)). While analyzing the mitochondrial OXPHOS complex I subunits, some of them were shown to be upregulated while some other subunits were downregulated. Eventhough we did not observe decreased or increased fatty acid-mediated complex I respiration in diabetic tissue, the mass spectrometry data indicated a deranged expression pattern of proteins which might affect the mitochondrial function in a later stage.

Figure 15.2.15. Mass spectrometry based cellular pathway analysis

A



Up regulated in Diabetic

6 Cluster(s)

Download File

Annotation Cluster 1	Enrichment Score: 5.55					Count	P_Value	Benjamini
KEGG_PATHWAY	Parkinson's disease	RT				22	5.6E-7	4.3E-5
KEGG_PATHWAY	Oxidative phosphorylation	RT				21	3.0E-6	1.2E-4
KEGG_PATHWAY	Huntington's disease	RT				25	4.2E-6	1.3E-4
KEGG_PATHWAY	Alzheimer's disease	RT				23	8.7E-6	2.2E-4
Annotation Cluster 2	Enrichment Score: 3.87					Count	P_Value	Benjamini
KEGG_PATHWAY	Fatty acid metabolism	RT				13	2.0E-7	3.1E-5
KEGG_PATHWAY	Valine, leucine and isoleucine degradation	RT				13	6.4E-7	3.3E-5
KEGG_PATHWAY	Propanoate metabolism	RT				10	1.3E-5	2.8E-4
KEGG_PATHWAY	Tryptophan metabolism	RT				10	8.9E-5	1.7E-3
KEGG_PATHWAY	Butanoate metabolism	RT				9	1.6E-4	2.7E-3
KEGG_PATHWAY	beta-Alanine metabolism	RT				7	4.6E-4	6.4E-3
KEGG_PATHWAY	Lysine degradation	RT				9	1.0E-3	1.2E-2
KEGG_PATHWAY	Limonene and pinene degradation	RT				4	2.7E-2	1.8E-1
KEGG_PATHWAY	Fatty acid elongation in mitochondria	RT				3	5.3E-2	3.1E-1
Annotation Cluster 3	Enrichment Score: 2.43					Count	P_Value	Benjamini
KEGG_PATHWAY	Metabolism of xenobiotics by cytochrome P450	RT				10	2.1E-3	2.2E-2
KEGG_PATHWAY	Drug metabolism	RT				10	2.6E-3	2.6E-2
KEGG_PATHWAY	Glutathione metabolism	RT				8	9.4E-3	8.2E-2
Annotation Cluster 4	Enrichment Score: 0.42					Count	P_Value	Benjamini
KEGG_PATHWAY	Dilated cardiomyopathy	RT				8	1.5E-1	5.8E-1
KEGG_PATHWAY	Hypertrophic cardiomyopathy (HCM)	RT				6	3.9E-1	8.4E-1
KEGG_PATHWAY	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	RT				3	8.9E-1	9.9E-1
Annotation Cluster 5	Enrichment Score: 0.35					Count	P_Value	Benjamini
KEGG_PATHWAY	GnRH signaling pathway	RT				9	9.9E-2	4.6E-1
KEGG_PATHWAY	Oocyte meiosis	RT				9	1.6E-1	5.8E-1
KEGG_PATHWAY	Long-term potentiation	RT				5	4.2E-1	8.4E-1
KEGG_PATHWAY	Melanogenesis	RT				5	7.1E-1	9.6E-1
KEGG_PATHWAY	Vascular smooth muscle contraction	RT				5	8.0E-1	9.8E-1
KEGG_PATHWAY	Wnt signaling pathway	RT				5	9.4E-1	1.0E0
KEGG_PATHWAY	Olfactory transduction	RT				3	1.0E0	1.0E0
Annotation Cluster 6	Enrichment Score: 0.33					Count	P_Value	Benjamini
KEGG_PATHWAY	Antigen processing and presentation	RT				7	2.1E-1	6.4E-1
KEGG_PATHWAY	Graft-versus-host disease	RT				3	5.7E-1	9.3E-1
KEGG_PATHWAY	Natural killer cell mediated cytotoxicity	RT				5	8.9E-1	9.9E-1

65 terms were not clustered.

B

Down regulated in Diabetic

DAVID Bioinformatics Resources 6.7
Laboratory of Human Retrovirology and Immunoinformatics (LHRI)

*** You are currently using DAVID 6.7. ***
*** If you are looking for DAVID 6.8, please visit our production site. ***

Functional Annotation Clustering

Help and Manual

Current Gene List: List_1
Current Background: Homo sapiens
429 DAVID IDs

Options Classification Stringency Medium

Rerun using options | Create Sublist

3 Cluster(s) Download File

					Count	P_Value	Benjamini
Annotation Cluster 1	Enrichment Score: 2.65						
KEGG_PATHWAY	Oxidative phosphorylation	RT			13	4.0E-4	5.0E-2
KEGG_PATHWAY	Parkinson's disease	RT			12	1.3E-3	7.8E-2
KEGG_PATHWAY	Huntington's disease	RT			14	2.3E-3	7.2E-2
KEGG_PATHWAY	Alzheimer's disease	RT			11	2.2E-2	4.3E-1
Annotation Cluster 2	Enrichment Score: 0.58				Count	P_Value	Benjamini
KEGG_PATHWAY	Cardiac muscle contraction	RT			6	7.9E-2	6.5E-1
KEGG_PATHWAY	Hypertrophic cardiomyopathy (HCM)	RT			4	4.6E-1	9.4E-1
KEGG_PATHWAY	Dilated cardiomyopathy	RT			4	5.1E-1	9.5E-1
Annotation Cluster 3	Enrichment Score: 0.13				Count	P_Value	Benjamini
KEGG_PATHWAY	Focal adhesion	RT			6	7.1E-1	9.9E-1
KEGG_PATHWAY	ECM-receptor interaction	RT			3	7.2E-1	9.9E-1
KEGG_PATHWAY	Small cell lung cancer	RT			3	7.2E-1	9.9E-1
KEGG_PATHWAY	Pathways in cancer	RT			8	8.6E-1	1.0E0

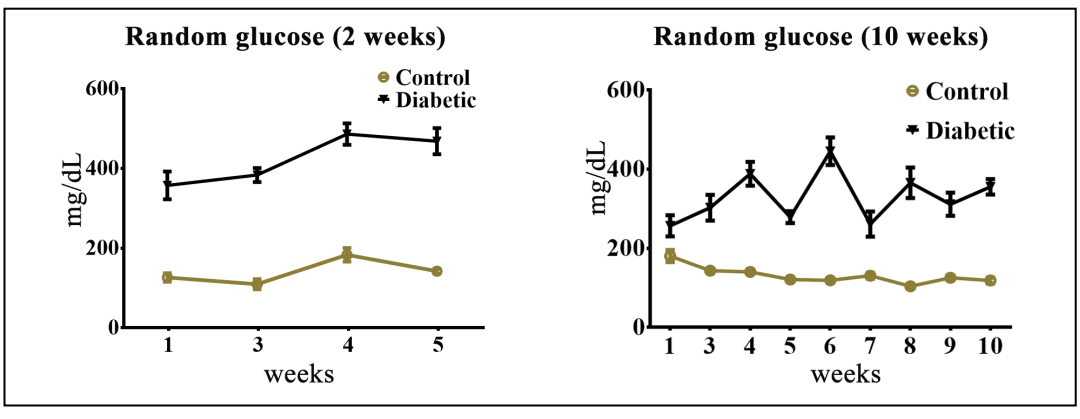
41 terms were not clustered.

(A) A list of pathways that were upregulated in diabetic cardiac tissue. (B) A list of pathways that were downregulated in diabetic cardiac tissue.

15.2.5.Development of T2DM mice model

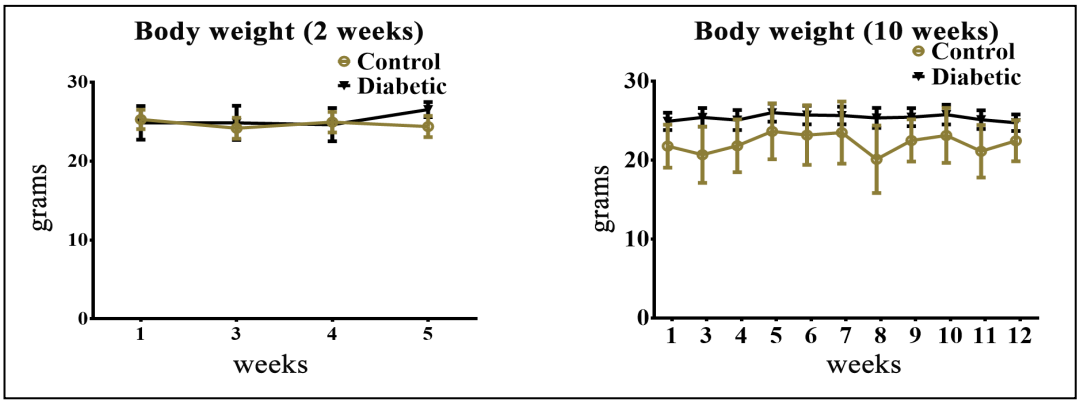
C57BL/6 mice were selected for creatingstreptozotocin (STZ)/ nicotinamide (NA)-induced type 2 diabetes mellitus model. The blood plasma parameters showed that STZ/NA-injected mice had significantly high glucose levels (Figure 15.2.16), while maintaining same body weight as the controls throughout the period of the study (Figure 15.2.17).

Figure 15.2.16. Random blood glucose measurements over time



The random blood glucose measurements over time were higher for diabetic mice at 2 weeks and 10 weeks. Error bars represent ± SEM. (n=6 in each group)

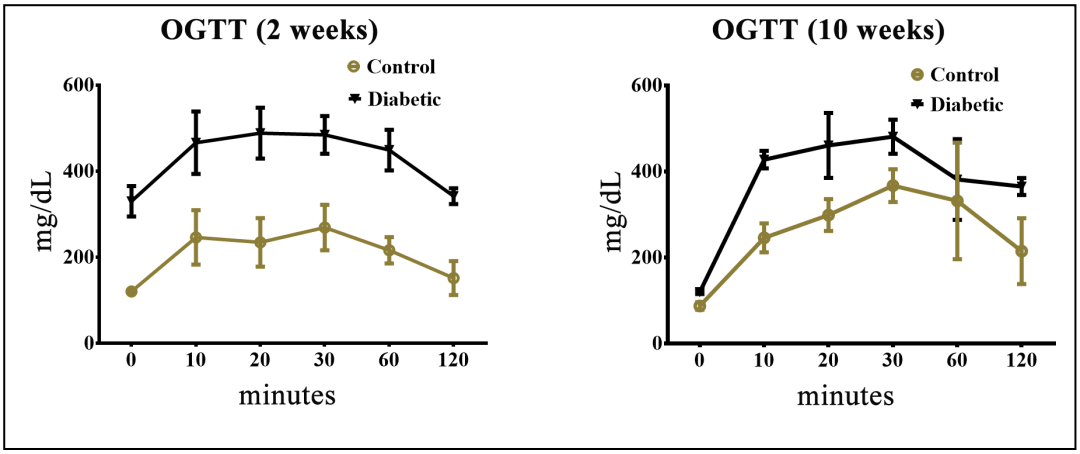
Figure 15.2.17. Body weight measurement over time



The body weight of diabetic mice wassimilar to that of the control mice at 2 weeks and 10 weeks. Error bars represent ± SEM.(n=6 in each group)

The oral glucose tolerance test showed that the blood glucose level after glucose load (a gold standard for detecting type 2 diabetes) was higher in diabetic mice than the control mice (Figure 14.2.18).

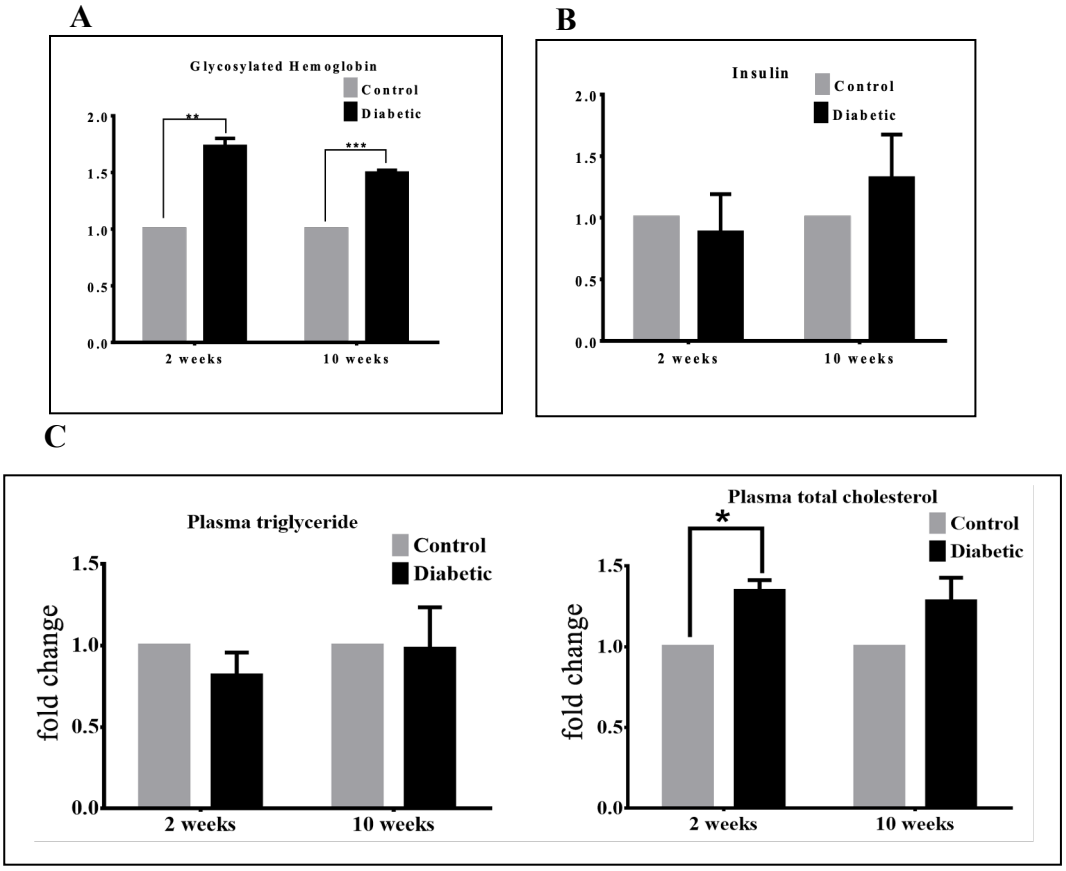
Figure 15.2.18. Oral glucose tolerance test



Higher level of plasma glucose was observed in diabetic mice on glucose load at both 2 weeks and 10 weeks.Error bars represent ±SEM.(n=6 in each group)

The glycosylated hemoglobin levels in diabetic mice were significantly higher than their corresponding controls (Figure 14.2.114(A)). The plasma insulin levels in diabetic mice were not significantly different from that of control mice and this indicated that the STZ/NA-induced diabetes mellitus was type 2 and not type 1 (Figure 14.2.114(B)). Other blood parameters like the plasma triglyceride level was not altered in diabetic mice while there was significantly higher levels of plasma cholesterol levels in diabetic mice at 2 weeks time point but not at 10 weeks (Figure 14.2.114(C)).

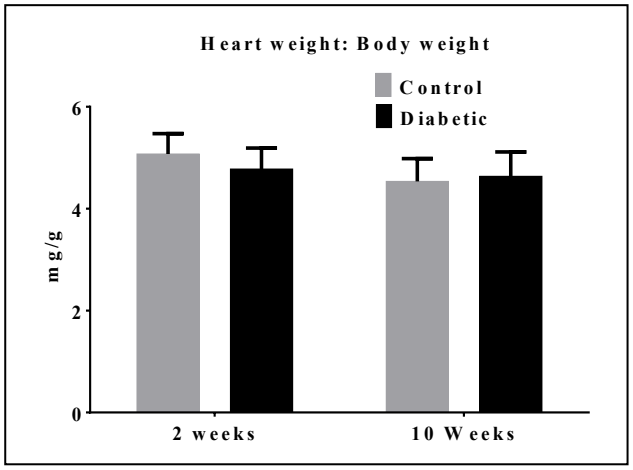
Figure 15.2.114. Blood plasma parameters



(A) Plasma insulin levels in STZ/NA-induced diabetic mice were similar to that of control mice. (B) Plasma triglyceride levels showed unaltered levels in diabetic mice while there was a significant increase in plasma cholesterol levels at 2 weeks time point. Error bars represent ± SEM.(n=6 in each group)

The heart weight: body weight ratio was calculated in diabetic mice at both time points and found that the values were similar to that of their corresponding controls (Figure 14.2.20). This indicated that no structural changes like hypertrophy/cardiomyopathy occurred in diabetic heart till 10 weeks of untreated diabetes.

Figure 15.2.20. Heart weight to body weight ratio



A representative bar graph showing the ratio of heart weight (milligrams) and body weight (grams) of mice at 2 weeks and 10 weeks of untreated diabetes. The error bars represent ± SEM.(n=6 in each group)

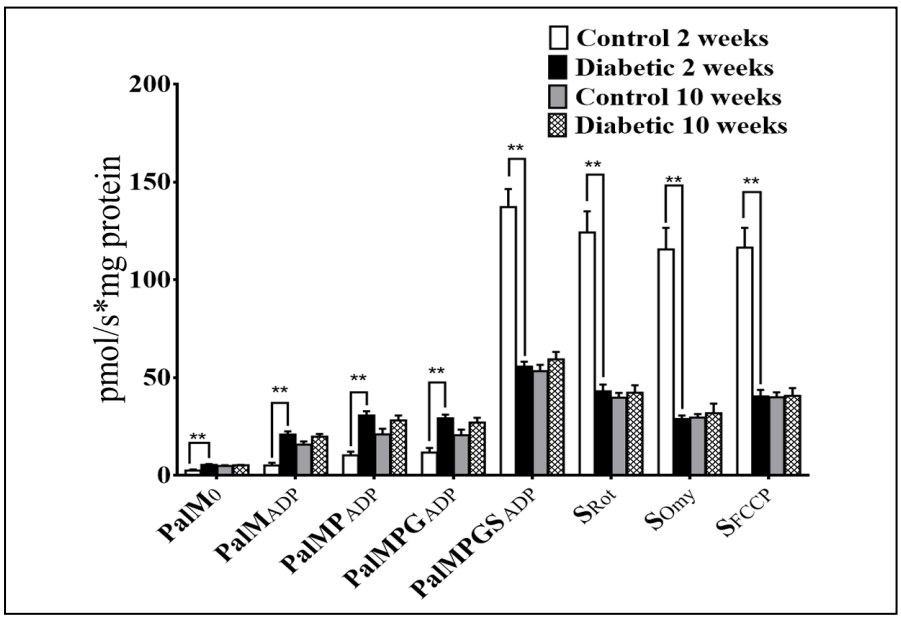
Findings:

- **STZ/NA-induced T2DM in C57BL/6 mice and hyperglycemia was maintained till 10 weeks, which was the longest duration of time selected for the present study**
- **Unchanged body weight and insulin levels showed that the diabetes mellitus developed in mice was of type 2**
- **Unchanged heart weight: body weight ratio showed absence of hypertrophy/cardiomyopathy at the specific time points**

15.2.6. Cardiac mitochondrial respiration

To determine whether T2DM alter cardiac mitochondrial function, we checked the mitochondrial respiration in isolated mitochondria by high-resolutionrespirometry. After the addition of 10µl aliquot of the mitochondrial suspension to each of the chambers, the sequential addition of palmitoyl L- carnitine, malate, ADP, pyruvate, glutamate, succinate, rotenone, oligomycin, FCCP, and antimycin showed significant increase in the state 2 and state 3-complex I and ETF respiration in 2 week diabetic mitochondria and significant lower oxygen consumption rate (OCR) by complex I +II respiration (state 3) of 2 week diabetic mice. Complex II alone (after addition of rotenone) showed significantly low OCR at 2 week time point. But it was observed that at 10 week time point the diabetic mitochondria showed no significant difference in any of the complex respiration (Figure 14.2.21). The OCR was expressed in pmol/s*mg of mitochondrial protein and the values are ROX (residual oxygen consumption rate) corrected. ROX represented the residual oxygen flux when electron transport is inhibited in the mitochondria by antimycin and the flux values are normalized by ROX.

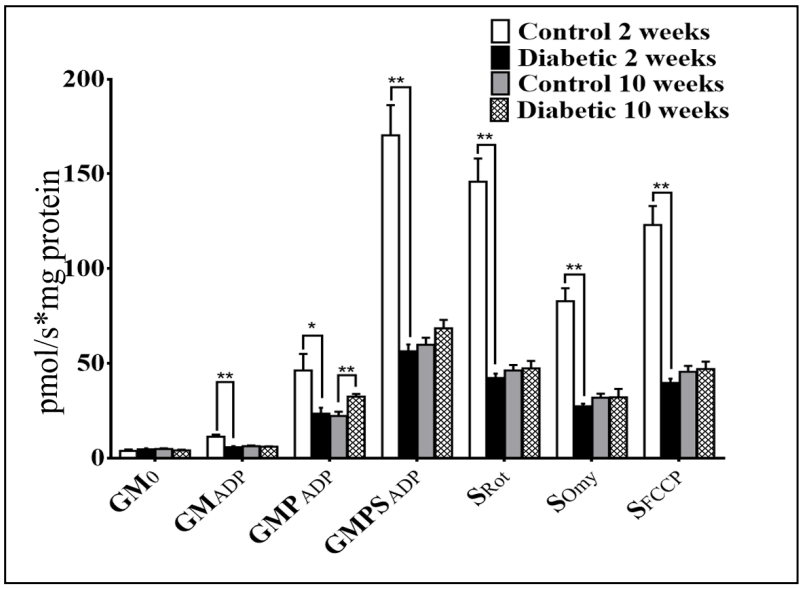
Figure 15.2.21. Increased mitochondrial fatty acid substrate utilization and decreased complex II-mediated respiration in diabetic mice



States of respiration of control and diabetic mitochondria (2 weeks and 10 weeks) with substrates palmitoyl L-carnitine followed by pyruvate, glutamate and succinate, inhibitors rotenone and oligomycin and the uncoupler FCCP. $PalM_0$- State 2 respiration, $PalM_{ADP}$- State 3 respiration of palmitoyl L- carnitine, PalMP- State 3 respiration of both palmitoyl L-carnitine and pyruvate, PalMPG- State 3 respiration of palmitoyl L-carnitine with pyruvate and glutamate, PalMPGS- State 3 respiration of palmitoyl L-carnitine with pyruvate, glutamate and succinate, S_{Rot} – State 3 respiration of succinate after inhibition of complex I activity by rotenone, S_{Omy}- State 4 respiration byoligomycin, where complex V activity is blocked. Values are represented as mean ± SEM. (p-value <0.05, ** p-value <0.01). (n=6 in each group)*

In other chamber, the sequential addition of glutamate and malate, ADP, pyruvate, succinate, rotenone, oligomycin, FCCP, and antimycin revealed significantly lower state 2, state 3-complex I, state 3-complex I +II and state 3 complex II (after addition of rotenone) respiration in 2 week diabetic mitochondria (Figure 15.2.22). The OCR was expressed in pmol/s*mg of mitochondrial protein. At 10 weeks of diabetes, significant increase in pyruvate-mediated complex I state 3 respiration was observed and unchanged OCR in glutamate-mediated complex I state 3 respiration and succinate-mediated complex II respiration.

Figure 15.2.22. Diabetic mitochondria show altered glutamate-mediated complex I and succinate-mediated complex II respiration

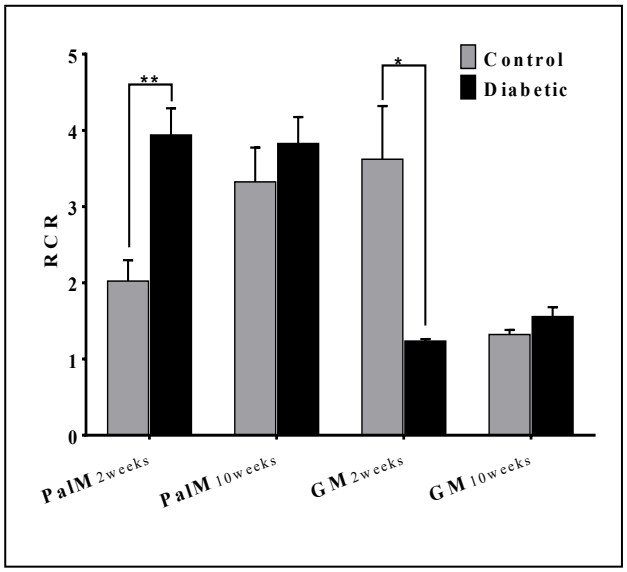


States of respiration of control and diabetic mitochondria with substrates glutamate, pyruvate and succinate, inhibitors rotenone and oligomycin and the uncoupler FCCP.GM_0- State 2 respiration, GM_{ADP}- glutamate-mediated state 3 respiration of complex I, GMP- glutamate and pyruvate-mediated state 3 respiration of complex I, GMPS- glutamate/pyruvate-mediated state3 respiration of complex I and succinate- mediated state 3 respiration of complex II, S_{Rot} – succinate-mediated state 3 respiration of complex II after inhibition of complex I by rotenone, S_{Omy}- State 4 respiration byoligomycin, where complex V activity is blocked.Values are represented as mean ± SEM. (p-value <0.05, **<0.01). (n=6 in each group).*

An increase in fatty acid-mediated mitochondrial complex I and ETF respiration and a decreased glutamate-mediated complex I respiration in 2-week diabetic mice wasevident when respiratory control ratio (RCR) was calculated, and there was no change in RCR in 10 week

diabetic (Figure 15.2.23). In summary, increased fatty acid substrate utilization clearly showed that 2 weeks of untreated diabetes has made immediate effects in mitochondrial substrate utilization by depending mainly on fatty acids, while at a 10-week time point, the diabetic cardiac mitochondria was responding to various substrates similar to that incontrols.

Figure 15.2.23. Respiratory control ratio



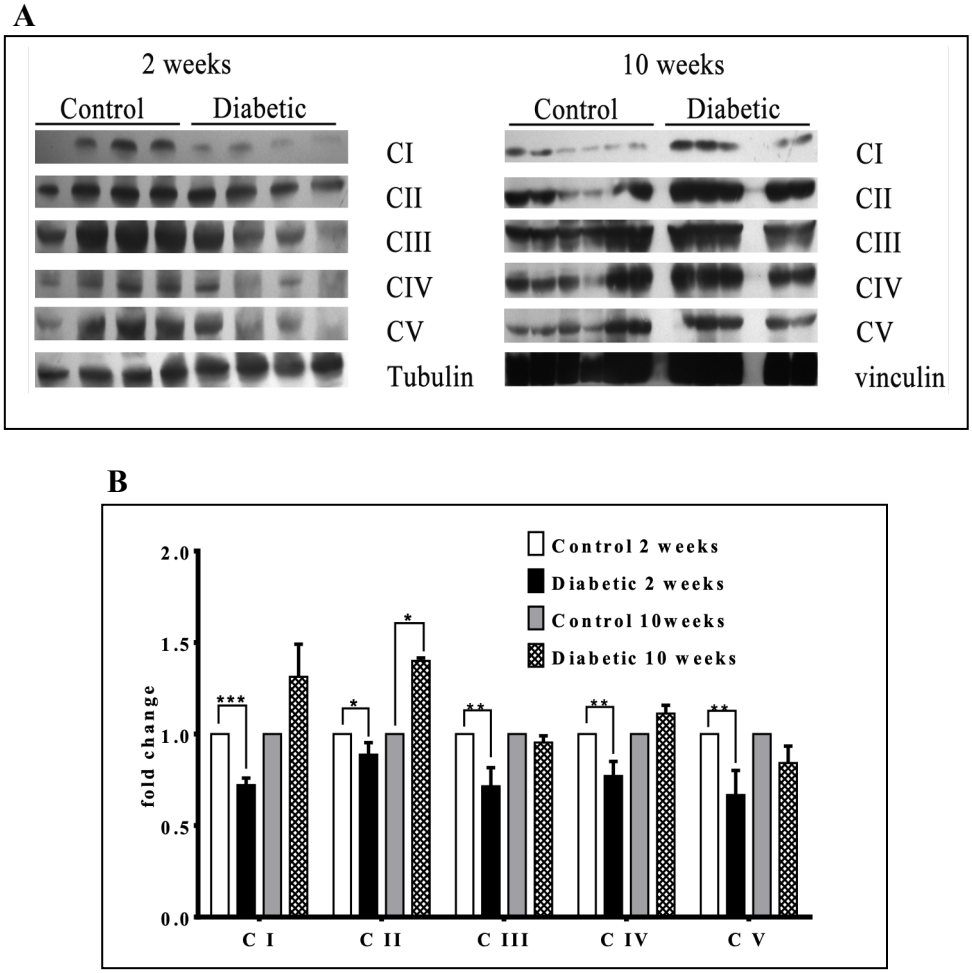
Palmitoyl L-carnitine-mediated RCR is shown to be increased in 2 week diabetic mice while glutamate-mediated RCR is shown to be decreased. No change was observed in RCR values in 10 week diabetic mice Values are represented as mean ± SEM.

Findings:

- **Increased palmitoyl L-carnitine-mediated complex I respiration in 2 week diabetic mice**
- **Unchanged palmitoyl L-carnitine-mediated complex I respiration in 10 week diabetic mice**
- **Increased pyruvate-mediated complex I respiration in 10 week diabetic mice**
- **Decreased succinate-mediated complex II respiration in diabetic mice at 2 weeks**
- **Increase in RCR in 2 week diabetic mitochondria when fatty acid was utilized while low RCR when glutamate was consumed**
- **Unchanged RCR at 10 weeks of T2DM when either of the substrates was consumed**

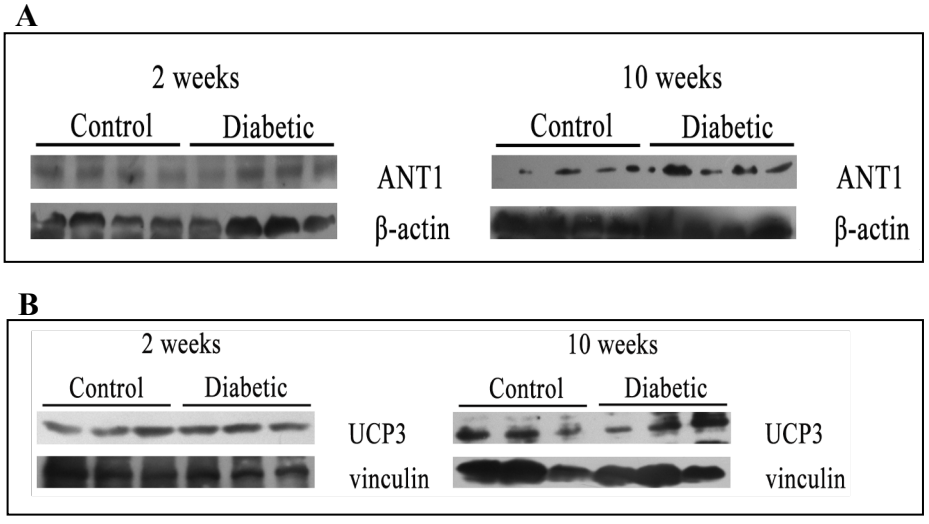
In order to check whether these changes in activity of complexes were due to the changes in their expression, we explored electron transfer chain complex levels in whole heart homogenates. There was significantly low expression of OXPHOS complexes in 2 week diabetic mice. Except complex II, none of the complex (I-V) levels varied much between the control and diabetic in 10 week diabetic mice (Figure 15.2.24). Other mitochondrial proteins UCP3 and the ANT1 were shown to be unchanged in expression in diabetic group (Figure 14.2.25).

Figure 15.2.24. Expression of oxidative phosphorylation (OXPHOS) complexes

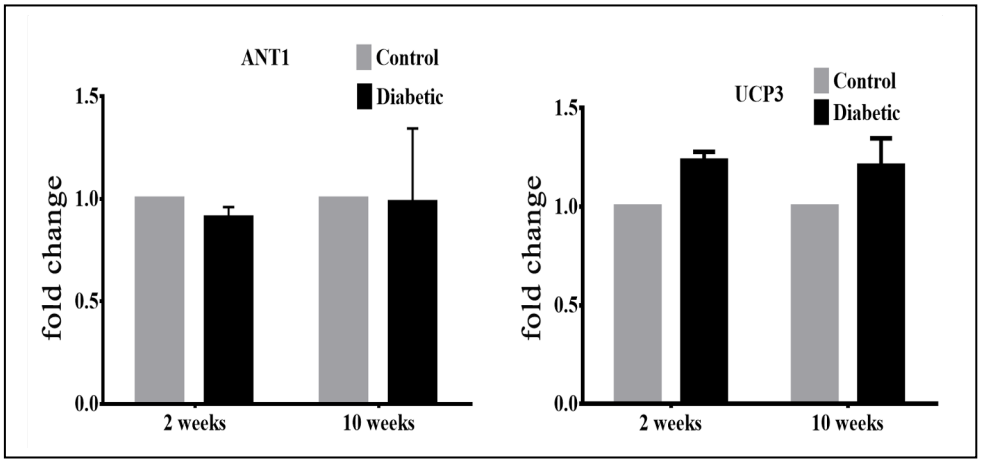


(A)Mitochondrial OXPHOS complex expression is decreased in 2 week diabetic heart as shown by western blot analysis. (B) A representative bar graph showing fold change in expression levels of OXPHOS complexes in diabetic cardiac tissue with respect to control at respective time points. Error bars represent ±SEM (n=6 in each group).

Figure 15.2.25: Expression of UCP3 and ANT1



C



(A and B) Unaltered expression of UCP3/ANT1 in diabetic cardiac tissue is showed by western blotting. (C)The representative bar graphs showing the fold change in expression of the respective proteins. Error bars represent ±SEM (n=6 in each group).

Findings:

- **Decreased expression of electron transport chain complexes in 2 week diabetic mice**
- **Increased expression of complex II in 10 week diabetic mice**
- **Unchanged expression of UCP3 and ANT1**

16. Contributions made towards increasing the state of knowledge in the subject:

16.1 Human study

As opposed to animal studies, cardiac mitochondrial function is not well explored in humans with T2DM. The aim of this study was to determine whether T2DM influence cardiac mitochondrial function in atrial appendage tissue obtained from patients undergoing elective coronary artery bypass graft (CABG) surgery. We hypothesized that diabetic cardiac mitochondria would show strong mitochondrial dysfunction, along with decreased fatty acid metabolism and elevated oxidative stress. This hypothesis is based on the fact that Asian Indians have unusual susceptibility to T2DM that contributes to cardiovascular complications(Wild et al., 2004)(Nair et al., 2008). As mentioned elsewhere, serious limitations to studying pathophysiology at the cellular level in human heart exist. Since biopsies of healthy human heart cannot be obtained, the issue of a true control remains for the study. Moreover, all the patients had underlying coronary artery disease and subsequent ischemic heart disease and were on numerous medications, which could alter mitochondrial function. We determined to follow a cross sectional study design, as done earlier by others (Montaigne et al., 2014)(Anderson et al., 20014) between diabetic and non diabetic patients with coronary artery disease and to keep both groups similar with respect to age, lipid levels, NYHA class, medications etc., while isolating a single clinical variable that is distinctly different between them. In this study, the single variable was hyperglycemia due to T2DM. With this approach,

it can be reasonably concluded that, differences are probably due to T2DM, and not one of the multitude of other clinical variables that exist in patient cohorts.

16.1.1. Human cardiac mitochondrial respiration

Our findings show no alterations in fatty acid substrate utilization in diabetic patients before the onset of clinical cardiomyopathy. Based on the mitochondrial respiration study in diabetic myocardium, we have not observed much difference in oxygen consumption occurring through complex I when palmitoyl L-carnitine was supplied. This is the first ever report on Asian Indian cardiac mitochondrial respiration and this is in contrast to the data published by Anderson *etal*and Montaigne *etal*, where they have noticed significantly low oxygen consumption rate when palmitoyl L-carnitine was provided to the diabetic human permeabilised cardiac fibres(Anderson et al., 20014)(Montaigne et al., 2014). This may be due to the difference in ethnicity/ NYHA class of human subjects included in the present study. Our study included Asian Indians belonging to NYHA class II. Also in Montaigne's study mitochondrial respiration was compared between diabetic and non diabetic patient groups of similar weight statuses(Montaigne et al., 2014), while in the present study the BMI status of diabetic patients differ significantly. Or else this discrepancy may be due to the difference in metabolic response between mitochondria in permeabilisedmyofibres and isolated mitochondria. Howerver, it is shown in our study that glutamate- mediated complex I respiration is low in diabetic mitochondria, which supports the findings of Anderson *etal*. The ambiguous nature of response of complex I towards two different substrate combinations indicates the necessity of analyzing the carbohydrate arm of cardiac metabolism too. It can also be interpreted in a way that the decreased glutamate-mediated complex I respiration may not be due to the decreased activity of complex I it might be due the decreased availability of NADH as a result of deranged activity of TCA cycle enzymes leading to improper substrate utilization in diabetic mitochondria. Inorder to confirm this argument, we have to analyze the TCA cycle enzyme activity. Irrespective of the presence or absence of fatty acids, succinate- mediated respiration, along with complex I substrates, was lower indicating possible decreased activity of complex II in diabetic subjects. But this decreased complex II respiration was not significant when oxygen consumption by complex I was inhibited by rotenone. Eventhough our data does not support increased fatty acid oxidation, the metabolic inflexibility of diabetic myocardium is manifested through deranged glutamate and succinate utilization, showing dependence on fatty acids rather than carbohydrates for ATP production. However, there was no difference in the coupling between oxidation and phosphorylation, as determined by RCR, in either palmitoyl L-carnitine- and glutamate-mediated oxygen consumption. The diabetic myocardial OXPHOS complex protein (CI –CV) expression levels and the mitochondrial content were also found it to be of no different from non-diabetic patients that indicated the effect of T2DM only on the activity of OXPHOS complexes regardless of their expression or the number of mitochondria.

16.1.2. Human cardiac fatty acid metabolism and its regulation

Since palmitoyl L-carnitine- mediated complex I respiration was seen unchanged between the groups, we checked components of fatty acid uptake, metabolism and its regulators. The current study demonstrated no change in levels of proteins involved in fatty acid uptake, CD36 and

FABP in diabetic cardiac tissue. Also levels of expression of one of the key enzymes involved in β- oxidation, long chain acyl CoA dehydrogenase (ACADL), was unaltered. Thus the uptake and utilization of fatty acids were not deranged in diabetic myocardium. Activity of ACADL is reported to be regulated by deacetylation by a family of NAD^+- dependent deacetylases namely, sirtuins. Earlier reports on increased acetylation of cardiac fatty acid enzymes was shown in high fat fed obese mice which suggested a critical role of acetylation control in heart failure related to obesity and diabetes (Alrob et al., 2014). Analysis of sirtuin levels revealed no significant change between the groups and further identification of unaltered mitochondrial acetylome and acetylated ACADL status in diabetic myocardium indicated that T2DM has not affected acetylation/deacetylation regulatory mechanism of fatty acid oxidation. Important nuclear regulators of fatty acid metabolism are PGC1α/β and PPARα. The unaltered levels of these nuclear regulators also corroborated the balanced fatty acid utilization in diabetic mitochondria. The intramyocardial triglyceride content of diabetic tissues was similar to that of non diabetic, once again emphasizing the occurrence of relatively unaffected fatty acid utilization in T2DM.

It was anticipated that in the incidence of metabolic inflexibility in diabetic myocardium, the expression of AMPK might be changed, which is an indicator of energy deficit in cells. But the levels of subunits of AMPK (α and β) and their phosphorylated forms were shown to be unchanged. Also the expression of UCP3, which uncouples fuel oxidation from conversion of ADP to ATP, resulting in decreased synthesis of ATP, in diabetic subjects was not significantly different from that of non diabetics, eventhough partial uncoupling of respiration from ATP production by upregulation of expression and activity of uncoupling proteins are reported in one of the earlier studies of diabetic models (Buchanan et al., 2005). These data together pointed out that the diabetic myocardial AMP: ATP ratio may not be much varied in diabetic cardiac tissue. The acetylation status of adenine nucleotide translocase 1 (ANT1) was unchanged although its expression level was significantly increased in diabetic myocardium, showing possible unchanged activity of ANT1 through acetylation/deacetylation axis of control, which in turn supports the optimum ATP demand in diabetic group.

16.1.3. Diabetic human cardiac oxidative stress

Compared with glucose oxidation, reliance on fatty acid oxidation for ATP production results in higher mitochondrial oxygen consumption, and the yield of ATP per oxygen atom consumed (P/O ratios) show that fatty acids are less efficient fuel. In due course, this leads to increased oxidative damage. According to published reports, human diabetic myocardium were shown to have high nitric oxide stress (Anderson et al., 20014).Also we have published earlier that untreated hyperglycemia for over a period of 14 weeks in swiss albino mice had increased levels of cardiac iNOS(Jayakumari et al., 2014). In contrast to these reports, current study shows relatively unaltered levels of 3 nitro tyrosine and iNOS levels demonstrating less nitric oxide- mediated stress in diabetic tissues. No change in expression of Mn-SOD and glutathione peroxidase (GPx), emphasized absence of overt oxidant stress. Nrf2 is the regulator of transcription of anti oxidant proteins and the absence of significant change in levels of expression of Nrf2 explained the unchanged expression of Mn-SOD in diabetic heart tissue.

Accordingly, no notable evidence was obtained with respect to increased myocardial oxidant stress. The possible explanation to this may be the absence of ischemic insult in the atrial appendage tissues or that the post translational modifications due to T2DM affecting the activity of crucial enzymes may be effectively reversed inorder to maintain homeostasis.

16.1.4. Streptozotocin-induced diabetic mice

The present study was done in order to check the effect of untreated T2DM for two different time periods of hyperglycemia (2 and 10 weeks (when compared to human life time, these time periods are equivalent to 1.7 years and 8.3 years of untreated T2DM) ("The Mouse in Biomedical Research, Volume 4 - 2nd Edition," n.d.) on cardiac mitochondrial respiration and to analyze the effect of resveratrol and honokiol, activators of sirtuins, in diabetic cardiac mitochondrial respiration and fatty acid metabolism at 2 time points of T2DM progression. To achieve this,C57Bl/6 mice, was selected and T2DM was induced by streptozotocin and nicotinamide. With this combination we were able to develop a non- obese type 2 diabetic model as described by Nakamura *et al* (Nakamura et al., 2006). Body weight, random blood glucose and oral glucose tolerance test (OGTT) data were analyzed and these revealed that the mice developed hyperglycemia and it was maintained for 10 weeks without any decrease or increase in body weight. Heart weight to body weight ratio was found to be similar in diabetic mice which demonstrated that at the time of euthanasia, the diabetic mice heart did not develop any structural changes. This model is similar to the human subjects included in the current study, where the diabetic subjects did not show major cardiac structural changes. Also the insulin levels of 2-week diabetic mice were maintained like the control group and an increase in its levels were observed at 10-week time point, although insignificant, indicated a trend towards development of hyperinsulinemia at a further extended time period of T2DM. Plasma triglyceride levels were unchanged in 2 week and 10 week diabetic mice while total cholesterol levels were found to be increased after 2 weeks of hyperglycemia and then maintained like control group at 10 weeks. Together these data showed the development of a non-obese T2DM model.

16.1.5. Mitochondrial respiration

The main objective of the present study with mice model was to analyze the cardiac mitochondrial function at different time points of untreated diabetes mellitus and evaluate the effect of resveratrol and honokiol in regulating diabetic mitochondrial respiration. For mitochondrial functional studies we adopted high-resolution respirometry and substrate-uncoupler-inhibitor-titration (SUIT) protocols of 2 different substrate combinations were used: 1) palmitoyl L-carnitine along with glutamate, pyruvate and succinate and 2) glutamate, pyruvate and succinate. By combining the fatty acid and carbohydrate substrates additive effect of these substrates on mitochondrial respiration could be analyzed and this represented a metabolic condition that prevails in T2DM where there is excess supply of fatty acids and glucose. Our study clearly demonstrated increased palmitate + glutamate and pyruvate utilization after 2 weeks of T2DM while glutamate and pyruvate utilization was decreased at 2 weeks in the absence of palmitate. Eventhough this seemed contradictory, it was observed that after palmitate addition no further increment in complex I-mediated oxygen consumption had

occurred by glutamate and pyruvate addition. Thus it was shown that 2 weeks of T2DM impaired glutamate and pyruvate mediated complex I respiration and thus cardiac tissue depended more on fatty acids. Another prominent finding was the decreased oxygen consumption rate (OCR) by complex II. The maximal respiratory capacity of succinate was also shown to be decreased in 2-week T2DM heart mitochondria by addition of uncoupler, FCCP. All these changes in 2-week diabetic mice mitochondria were not observed in 10-week diabetic mitochondria. The 10-week T2DM mitochondria were showing similar oxidative capacity as control mitochondria, eventhough pyruvate- mediated complex I OCR was increased. The respiratory control ratio (RCR) showed increased fatty acid-mediated mitochondrial complex I oxidative capacity and decreased glutamate-mediated complex I oxidative capacity at 2 weeks. After 10 weeks of hyperglycemia, T2DM heart mitochondria did not show any further increase or decrease in RCR by fatty acid and glutamate utilization. Previous reports on different models of T2DM showed varying data on mitochondrial fatty acid and carbohydrate utilization. Boudina*etal* showed unchanged palmitoy L-carnitine- mediated complex I respiration in ob/ob mice after 14 weeks, where as Buchanan *et al* showed increased palmitoyl L-carnitine-mediated complex I respiration in db/db after 8 weeks (Boudina et al., 2005)(Buchanan et al., 2005). Lou *et al* reported a similar data as shown by Buchanan *et al*, i.e., an increased palmitoyl CoA/carnitine/malate- and octanoylcarnitine/malate-mediated complex I respiration in fructose-fed T2DM rat heart after 6 weeks (Lou et al., 2017). With regard to carbohydrate substrates, ob/ob and db/db T2DM mice showed decreased pyruvate-mediated complex I respiration and glucose oxidation respectively (Buchanan et al., 2005)(Boudina et al., 2005). Also a decreased RCR by glutamate-mediated complex I respiration was reported in db/db mice after 10 weeks (Tocchetti et al., 2012). Lou *et al*reported unchanged complex I respiration mediated by pyruvate, glutamate and succinate in fructose-fed rats (Lou et al., 2017). The published reports on various experimental models of T2DM were analyzed after different time periods of T2DM progression bringing high variation in end results. The present and previous study results on mitochondrial oxygen consumption rate necessitate analyses of mitochondrial respiration of multiple models of T2DM at multiple time points of hyperglycemia.

To verify whether the changes in activity are due to the difference in the expression levels of these complexes, we did western blot analyses of all five complexes (1-V). There was significant decrease in expression levels of all five complexes in 2 week T2DM mice heart while there was increase in complex II level and unchanged expression in all other complexes after 10 weeks of hyperglycemia. Even though complex I expression was low in 2 week diabetic mitochondria; its oxygen consumption rate was high as evidenced by palmitoyl L-carnitine-mediated state 3 complex I respiration. Inspite of increase in complex II expression, complex II- mediated respiration was not increased in 10 week diabetic mice heart, indicating the fact that T2DM impaired the activity of complex II.This demonstrated that T2DM influenced complex activities independent of their expression levels. There could be other reasons like T2DM-induced oxidant-mediated posttranslational modifications that might have oxidized the amino acid residues in complexes, leading to impaired oxygen consumption. The expression of UCP3 and ANT1 were unchanged in diabetic mice at both time points. These data together showed that eventhough there was impaired carbohydrate-mediated complex I

respiration as a result of a sudden hyperglycemic insult, heart met its energy demand by relying more on fatty acids as evidenced by high fatty acid dependent RCR in 2-week diabetic mice cardiac mitochondria. With time, the heart showed a trend towards adaptation to the hyperglycemic environment that we observed unchanged fatty acid- and glutamate- dependent RCR in 10-week diabetic mice.

17. **Conclusions summarizing the achievements and indication of scope for future work:**

17.1. Human study

The present study reports are in contrast to the available human data published previously as our data pointed towards the absence of effect of T2DM on cardiac fatty acid oxidation, mitochondrial function and associated oxidative stress. With respect to our study, even if we observed a significant decrease in glutamate- mediated respiration, the overall energy and stress status of diabetic myocardium was comparable to that of non diabetic myocardium. Nair etal, in their study exploring skeletal muscle mitochondrial function in Asian Indians have shown that their mitochondrial function was comparable between diabetic and non diabetic, and despite the unusual susceptibility of Asian Indians towards T2DM, had higher OXPHOS capacity than Northern European Americans (Nair et al., 2008). When compared with the previous reports on other ethnic groups, the current study showed that Asian Indian population showed relatively unaffected cardiac mitochondrial fatty acid utilization, while the reason behind this needs to be deciphered.

17.2. Mice study

The current study reported that the T2DM cardiac mitochondria respond diversly at different time points of T2DM. Also it responded differently to distinct combinations of substrates. The 2 week diabetic mitochondria showed enhanced palmitoyl L-carnitine- and reduced glutamate-mediated complex I respiration. The varying expression profile of electron transport chain complexes at 2 different time points of T2DM conveyed that T2DM affected the activity of these complexes independent of their quantity in mitochondria.

17.3. Scope for future work:

17.3.1. Human study

Since the present study included non diabetic human subjects those had ischemic injury, a future study can be undertaken by collecting atrial appendage from patients other than ischemia, for example, patients with septal defect, and studying their mitochondrial function will definitely provide a better insight into the actual role of type 2 diabetes mellitus on cardiac mitochondrial function.

17.3.2. Mice study

Our present type 2 diabetic mice model was developed by injecting streptozotocin and nicotinamide, which enabled us with a hyperglycemic condition, that mimicked the hyperglycemic conditions prevailed in type 2 diabetes mellitus. But the hyperlipidemia, which is another hallmark feature of type 2 diabetes was not developed in our diabetic model. Thus it necessitates a high fat fed sptreptozotocin-induced diabetic model and a similar study can be performed in this model at different stages of disease progression.

18.Science and Technology benefits accrued:

a. List of Research publications with complete details:

- Nandini R J, Raji S R, Ashok S, Surabhi SV, Saurabh N, Manjunatha S, Vivek V Pillai, Jayakumar K, Srinivas Gopala. Impaired substrate mediated cardiac mitochondrial complex 1 respiration with unaltered regulation of fatty acid metabolism and oxidative stress status in type 2 diabetic Asian Indians. **J of *Diabetes*** 12 (7): 542-555**,** 2020. **IF: 4.008**
- Anand CR, Bhavya Bharathan, Jayakumar K, Harikrishnan VS, Srinivas Gopala. Inorganic nitrite alters mitochondrial dynamics via Drp1 activation without modifying cell death and mitochondrial respiration in cardiomyoblasts under hyperglycemia. ***Toxicology In Vitro*****,** 70: pp-pp**,** 2021). **IF: 3.500.**
- Nandini Ravikumar Jayakumari, Raji Sasikala Rajendran, Ashok Sivasailam, Sulfath Thottungal Parambil, Anand Chellappan Reghuvaran, Harikrishnan Vijayakumar Sreelatha, Srinivas Gopala. Honokiol regulates mitochondrial substrate utilization and cellular fatty acid metabolism in diabetic mice heart. ***Eur J Pharmacol***, 896: pp-pp, 2021) **IF: 5.195**
- Raji Sasikala Rajendran, Nandini Ravikumar Jayakumari, Ashok Sivasailam, Anand Chellappan Reghuvaran, Vivek Velayudan Pillai, Jayakumar Karunakaran, Harikrishnan Vijayakumar Sreelatha, Shankarappa Manjunatha, Srinivas Gopala. Diminished substrate-mediated cardiac mitochondrial respiration and elevated autophagy in adult male offspring of gestational diabetic rats. ***IUBMB Life*** 73: 676-689, 2021. **IF: 4.709**
- Mahalaxmi Ganjoo, Ashok Sivasailam, Arun Gangadharan, Srinivas Gopala. Albumin binds to uncoupler CCCP to diminish depolarization of mitochondria. ***Toxicology In Vitro*** 80**:** 105325, 2022. **IF: 3.685**

b. Manpower trained on the project:

i. Research Scientists or Research Associates **:** Nil

ii. No. of PhD's produced **:** 3

iii. Other Technical Personnel trained **:** 2

c. Patents taken, if any **:** Nil

d. Products developed, if any **:** Nil

21. Procurement of/Usage of Equipment:

a. Details of Equipment:

Sl.No.	Name of equipment	Make/Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	Oxygraph	Oroboros 2 K	18.67 lakhs	30 July 2015	90%	Working properly

b. Suggestions for disposal of equipment(S): Presently working condition

(Name and Signature of PI)