5. Discussion

This study depicts for the first time a comprehensive list of the proteins and functional processes involved during post myocardial infarction remodeling, at early and late phases, in both the acutely stressed and minimally stressed regions of the heart. Major proteins and cellular networks altered with respect to the differential oxidative stress incident on the myocardium during the remodeling process have been determined by differential proteomic profiling, using iTRAQ technique. This has enabled us to compare and contrast the changes at the protein level occurring in the cardiac tissue as a whole, during the remodeling process.

5.1 Changes in myocardial structure during MI and post MI remodeling

Considering the structure of the myocardial sections derived from the infarct and non-infarct zones of the rat hearts during MI, with respect to control it can be clearly observed that the infarct zone suffers from acute deleterious alterations such as formation of second and third order waves, loss of intercellular matrix, while the alterations are milder at the non-infarct zone during MI. During 30 days post Mi remodeling, the infarct zone recovers the closely packed arrangement of cardiomyocytes even though second order waves are present. The non-infarct zone at 30 days post MI showed almost undisturbed rows of cardiomyocytes with only first order waves in individual cells. During the late post MI phase (120 days post MI), the closely packed and linear arrangement of cardiomyocytes rows is restored in the infarct zone with lengthening and thinning of individual cells compared to control. In the non-infarct zone there is no significant variation in the arrangement and structure of the cardiomyocytes compared to control sections. The histological study elaborates the fact that progressive alterations in myocardial organisation and structure occur continuously over the early phase till the late phase of remodeling, post MI, to restore the tissue to control like condition with minor changes such as lengthening and thinning of cardiomyocytes.

5.2 Compromised cardiac function during post MI remodeling

M-mode echocardiography data has elucidated the functional efficacy of the heart during the post MI time points considered, along with the structural changes brought about. It can be seen that MI cannot be identified from control condition by M-mode echocardiography i.e. it cannot be distinguished from a control condition heart in terms of left ventricular diastolic dimension, ejection fraction and fractional shortening. At 30 and 120 days post MI time points however, the M-mode parameters show significant changes from both control and MI conditions. The left ventricular end diastolic diameter and end diastolic volume have been progressively increased which indicates an enlargement in the chamber size that correlates with hampered pumping of blood. The ejection fraction which denotes the fraction of blood successfully pumped out by the ventricle (left) and thus measures the functional pumping efficiency of the heart has been
progressively decreased in the two post MI time points. The fractional shortening, a measure of the contractility of the cardiac muscle, has also been progressively lowered in the post MI time points compared to either control or MI conditions. Taken together, the M-mode data indicates comprised cardiac functioning and a change in its structure from control conditions and may qualify for a dilated cardiomyopathy phenotype, the latter being characterised by increased LVDD without an increase in wall thickening, decreased EF and FS.

Once the functional changes occurring during post MI remodeling had been assessed, the detailed proteome profile was elaborated by iTRAQ proteomics and analysed. An extensive list of proteins has been compiled from the proteomics data which examines the post MI phenomenon through the following aspects- differential protein expression at both the regions of acute stress as well as the non stressed, remote region; pursuing the difference in proteome profile at these two spatial regions over early and late time points. This has been done in order to acquire a comprehensive picture of the functional networks involved during post MI cardiac remodeling and enable us to compare and contrast the changes in the cardiac tissue as a whole.

5.3 Proteomic profile during myocardial infarction

We have analysed the proteomics data with MI as the fulcrum. During MI, at both the infarct and non-infarct zones stress response proteins, chaperones and proteins of the immune system showed upregulation exclusively. On the other hand, proteins involved in myofibril formation, contraction and organisation were downregulated exclusively. These data are in accordance with other studies (Fishbein et al., 2003; Wu and Ford, 1999) and indicate sarcomeric destabilization during MI.

`Metabolic proteins` was the major functional group with altered expression during MI, at both the spatial regions of the heart, compared to control. Proteins of fatty acid oxidation, especially β oxidation, and those of glucose oxidation were predominantly downregulated. In addition, intermediary metabolism and energy production were hampered due to downregulation of proteins involved in malate regulation, phosphocreatine cycle and lactate- pyruvate interconversion cycle. In our study, we have found the decreased expression of Lactate dehydrogenase B chain in the infarct zone. This may explain the lactate build-up and leakage previously reported in patients during MI (Schmiechen et al., 1997; Gatien et al., 2005). It can also result in inhibition of pyruvate supply to the citric acid cycle in cardiomyocytes. To complete the picture of absolute energy crisis in the myocardium during MI, we have found proteins of the citric acid cycle, electron transport chain and ATP synthesis, such as, Aconitate hydratase, Isocitrate dyhydrogenase (NADP+) and Ubiquinone biosynthesis protein COQ9 to be downregulated significantly.
The above data regarding MI points to the interesting fact that, despite levels of stress incurred by the two spatial regions being disparate, instead of being distinct, the protein profile that underwent changes in response to the stress was similar albeit different in amplitude.

5.4 Proteomic profile during post MI remodeling

Comparing the protein profile at 30 and 120 days post MI at the damaged and remote regions with respect to MI at the other end of the fulcrum, a few characteristic patterns emerged from this study. The stress response and chaperone system as well as immune system proteins that were directly induced by oxidative stress levels (Benjamin and McMillan, 1998; Pinckard et al., 1975), were downregulated at both the spatial regions, in direct reversal to the condition during MI. As oxidative stress levels decreased during post MI, the expressions of these proteins too were downregulated in a similar fashion which might explain the reversal.

The profoundly affected group of ‘metabolic proteins’ showed characteristic patterns of expression during the post MI time points. In the damaged zone, the proteins for glucose metabolism and β oxidation of fatty acids were upregulated along with those of malate regulation, phosphocreatine cycle and intermediary metabolism. Proteins of electron transport chain and oxidative phosphorylation were also upregulated. In the remote zone, on the other hand, proteins for amino acid metabolism, especially branched chain amino acid metabolism, were highly upregulated along with those of intermediary metabolism, citric acid cycle and electron transport chain. Thus there seems to be separate branches of metabolism (β oxidation vs branched chain amino acid metabolism) predominantly upregulated in the two spatial regions of the heart post MI, differentiating their response to MI over time.

In our study, we have found a unique expression pattern for the structural protein Dystrophin. It is a part of a multi protein complex that ensures proper organization and connectivity of intracellular myofibrils to the extracellular matrix, thus forming costameres (Anastasi et al., 2009). Dystrophin and its associated proteins are most often identified with muscular dystrophy related cardomyopathies (Finsterer and Stöllberger, 2003). During MI, Dystrophin was downregulated in the infarct zone while during 30 and 120 days post MI it was progressively and substantially upregulated. There was no appreciable change at any time in the remote region. Such a difference in expression distinguished the response profile of the two spatial regions of the heart during the remodeling process.

5.5 Cytoskeletal proteins during post MI remodeling

The interesting element revealed by this study was the unique expression pattern of cytoskeletal proteins in the infarct and remote zones during MI and post MI remodeling process. Intermediate filaments Desmin and Vimentin and microfilament forming βactin (non myofibrillar) were all upregulated during MI in both the cardiac regions. A few other studies
have found the same to be true and attributed this increase to the myocyte’s inclination to mitigate the effects of oxidative stress and preserve cellular architecture. During 30 and 120 days post MI, all three proteins showed progressive downregulation in the damaged zone while in the remote zone, only Vimentin expression was decreased. Again, such a difference in expression pattern has helped to distinguish the response profile of the two spatial regions of the heart during the remodeling process.

Though the behaviour of the stress responsive proteins and immune system proteins may be explained on the basis that, their alteration of expression is a direct consequence of the level of oxidative stress incident on the myocardium, similar explanation with respect to the intermediate filaments is insufficient. Differential expression pattern of the cytoskeletal proteins may represent cellular remodeling of the cardiomyocytes in the damaged zone that may result in compromised cardiac function.

5.5.1 Desmin aggregation during post MI remodeling

Further studies were done with Desmin to reveal that its expression followed the pattern of upregulation during MI and significant decrease during the post MI time points in the damaged region, as specified by the proteomics data. In addition, cleavage products of Desmin were clearly visible during post MI while they were absent either during MI or in control condition. Since cleaved Desmin has been found to form aggregates in other cardiomyopathies (Chen et al., 2003), we evaluated the same in our case.

Our filter trap assay revealed insoluble Desmin aggregates in the damaged region of the heart during the post MI time points in comparison to either MI or control group. To reveal the causative factor behind Desmin cleavage and aggregation, we performed studies on neonatal rat cardiomyocytes using a hypoxia-reoxygenation model as the closest possible simulation of our post MI remodeling groups. Western blot data again confirmed the loss of Desmin expression and presence of cleavage products at 8hr and 16hr reoxygenation. Immunofluorescence studies confirmed the presence of perinuclear Desmin aggregates at the reoxygenation time points studied.

It is known that Caspase3, a potent protease activated during MI, via Caspase6 can cleave Desmin (Chen et al., 2003). In our post MI groups as well as reoxygenation studies we found negligible cleavage of Caspase3 that would indicate its inactivity in these conditions. Instead, we found the increased activity of neutral protease Calpain1, another known mediator of Desmin cleavage, in both our post MI groups and in vitro studies. Calpain1 activity was confirmed by the generation of distinct and specific cleavage products of PKCa in the post MI groups. In vitro, the reoxygenation groups subjected to Calpain1 inhibition by ALLN showed the absence of perinuclear Desmin aggregates and decreased the expression of cleavage products.
products. It established that Calpain1 mediated Desmin cleavage and aggregation after hypoxia, during the reoxygenation time points studied. We may consider that Calpain1 is similarly responsible for Desmin cleavage and aggregate formation at the damaged region of the heart during the post MI time points studied. It represents a characteristic cellular remodeling of cardiomyocytes post myocardial infarction that may hamper the normal functioning of the heart. Further studies are needed to confirm whether such cellular remodeling is a causative factor for compromised cardiac function and heart failure that occurs after myocardial infarction. These observations indicate the molecular and cellular remodeling occurring in the heart after infarction and may advance the way for further studies that connect such remodeling precisely to the cardiac dysfunction and heart failure developing post myocardial infarction.

5.6 Metabolic remodeling of the heart after myocardial infarction

As previously mentioned, our proteomic analysis found proteins of the cardiac metabolic machinery to be profoundly altered at all stages, from MI to post MI remodeling. Therefore we assessed the total ATP content at the infarct and non-infarct zones to determine the status of the principle energy currency in the myocardium.

Total ATP content was decreased significantly in the infarct zone during 0 day MI compared to control which correlates to the proteomic analysis of concerted decrease of glucose and fatty acid oxidation enzymes in this study group. During post MI time points, at the infarct zone, a considerable variation was observed in the total ATP content. At 30 days post MI, the total ATP content was restored to almost the level of control group from the severe decrease seen during 0 day MI that again correlates to the proteomic finding of increased expression of metabolic enzymes in the group. In contrast, the total ATP content at 120 days post MI in the infarct zone was decreased significantly compared to control, even though no significant down regulation of metabolic enzymes have been elaborated in the proteomic analysis in this study group. In case of the non-infarct zone, there was considerable decrease in total ATP content at 0 day MI compared to control, however, during both 30 and 120 days post MI, the total ATP content recovered to control levels, correlating with the decrease and subsequent increase of metabolic enzymes in these study groups as evaluated from the proteomic study.

5.6.1 Mitochondrial dysfunction leads to energy depletion during late phase post MI remodeling

While the decrease of total ATP during MI and end stage heart failure has been reported previously (Mitra et al., 2015), decreased ATP content during remodeling has not been documented. We evaluated the activity of mitochondrial respiratory complexes I, II, III, IV and V which are directly involved in electron transport mechanism and generation of ATP (Wittig et al., 2008). Except for complex III, which could not be evaluated in our study, all the other
complexes showed varied levels of decreased activity in the infarct zone at 120 days post MI compared to either control or 0 day MI. This indicated that a dysfunction of the mitochondrial respiratory chain and complexes may be responsible for the decrease in total ATP content during late phase post MI remodeling.

From our proteomics data we found HSD17B10 gene or ERAB protein to be the only metabolism related candidate to be downregulated at the infarct zone during 120 days post MI remodeling. Previous studies have shown HSD17B10 to be an integral part of mammalian protein-only RNase P, an essential mitochondrial RNA processing enzymatic complex in addition to being a hydroxyl steroid dehydrogenase enzyme (Vilardo et al., 2012). HSD17B10 as a part of RNase P is designated as MRPP2 (Mitochondrial RNase P protein) along with MRPP1 and MRPP3 which catalyse the processing of the 5’ end of precursor mitochondrial tRNAs (mt tRNA) to mature them to functional tRNAs (Deutschmann et al., 2014) which are essential for mitochondrial protein translation. Mutation or knock down of HSD17B10 (MRPP2) has been associated with accumulation of precursor tRNAs and dysfunction and diminished activity of mitochondrial respiratory complexes-I, III, IV and V as they have multiple protein sub units encoded by the mitochondrial genome (Chatfield et al., 2015).

We evaluated the HSD17B10 protein levels in our infarct zone study groups of Mi and post MI time points. At 30 and 120 days post MI, the protein expression was significantly decreased compared to control. We next evaluated the status of precursor and total mt tRNA levels for three amino acids lysine, leucine and methionine. It was found that at 120 days post MI, in the infarct zone, all the precursor mt tRNAs showed significantly high accumulation compared to control. When the ratio of precursor to total (both processed and unprocessed) mt tRNAs for lysine, leucine and methionine were analysed it was found that at 120 days post MI remodeling period it was the highest compared to control or to any other time point studied.

Taking the results in consideration, it may be concluded that the decrease in total ATP content observed during 120 days post MI remodeling in the infarct zone is a consequence of two factors. One factor is the loss of abundance and activity of complex II during the 120 days post MI time point, a separate causative agent for ATP loss from the mt tRNA processing and protein translation dysfunction as complex II is not encoded by the mitochondrial genome. The other factor is the dysfunctional mitochondrial translation machinery due to accumulation of unprocessed precursor tRNAs as a result of decreased expression of HSD17B10 (MRPP2), an essential part of mitochondrial RNase P.