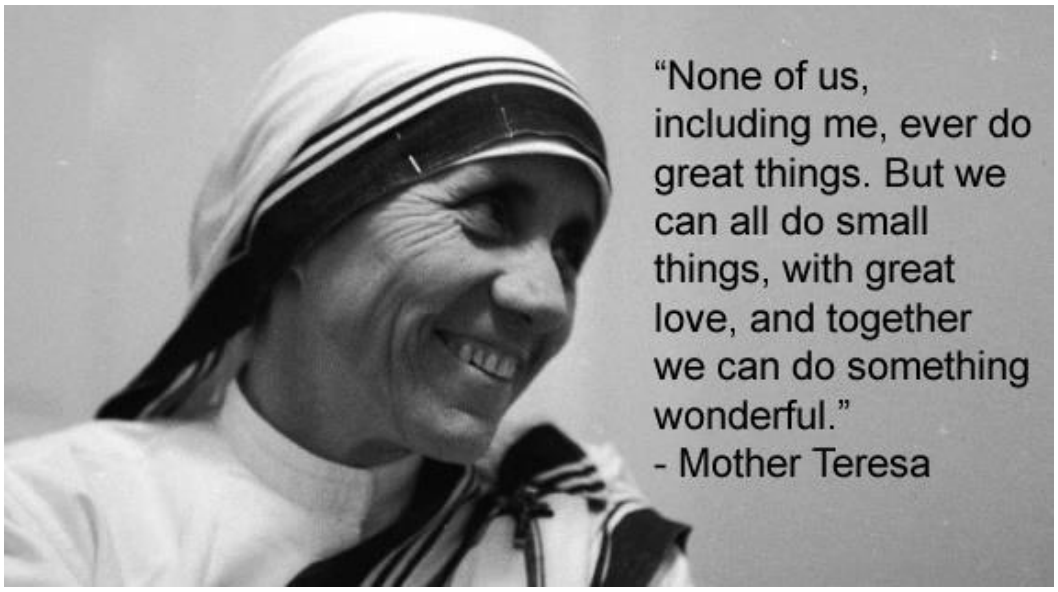

Epilogue

Character cannot be developed in ease and quiet. Only through experience of trial and suffering can the soul be strengthened, ambition inspired, and success achieved -Helen Keller

SUMMARY:

Oral cancer is the challenging health problem worldwide. In India, current rising trend in its incidence among younger population and its late diagnosis are the major challenges. Therefore, the use of cost effective, non-invasive and easy to use molecular markers for early detection is the need of the day. Alterations in glycosylation have long being associated with malignant transformation and metastasis. There is lack of reports on salivary glycosylation changes in patients with OPC and oral cancer patients. Thus, the present study most appropriately compared glycosylation changes from serum and saliva in patients with OPC, oral cancer patients and post-treatment follow-ups to establish its usefulness in early detection, prognostication and disease monitoring. Based on earlier reports (Yuwana *et al*., 2011; Kaur *et al*., 2013; De Freitas Silva *et al*., 2013), it was hypothesized that increase in MMPs might cause loss of E-cadherin in OPC at an early stage. Therefore, there is an urgent need for simultaneous evaluation of glycosylation, EGFR, E-cadherin, MMPs and c-Jun (mRNA and protein) in oral cancer patients. Hence, the present study evaluated glycosylation changes and MMPs (from blood and saliva), along with the pEGFR, *CJUN* and *ECAD* mRNA as well as protein expression from malignant and adjacent normal tissues to understand the pathway for c-Jun protein increase in oral cancer.

Noteworthy observations:

- A significant increase in 192 kDa, 170 kDa, 116 kDa and 44 kDa serum glycoproteins was observed in patients with OPC and oral cancer patients as compared to the controls. A 230 kDa was present in majority of the TH and an increasing trend was observed from TH controls to TH patients with OPC to TH oral cancer patients. This highlights the association of the 230 kDa band in tobacco related progression of oral cancer.
- An increasing trend in serum and salivary TSA/TP ratio was observed from controls to patients with OPC to oral cancer patients. Serum and salivary levels were found to be higher in patients with LN metastasis when compared to patients without LN metastasis. The salivary TSA/TP ratio was found to be

increased in patients with advanced disease as compared to those with early disease. It was observed that serum and salivary TSA/TP ratio was found to be higher in NR as compared to PT levels. It was observed that the values above cut-off of serum TSA/TP ratio was significantly associated with lower overall survival. Also higher values of salivary TSA/TP were associated with lower overall survival.

- Serum and salivary sialidase activities were found to be significantly elevated in oral cancer patients as compared to controls and were also higher in oral cancer patients as compared to patients with OPC. The results depicted higher levels of serum and salivary sialidase activities in patients with LN metastasis as compared to patients without LN metastasis. An increasing trend of both serum and salivary sialidase activity was observed from stage I to stage IV of the disease. Serum and salivary sialidase activity was observed to be decreased in CR as compared to PT levels, while in case of NR the levels were found to be increased.

- An increasing trend of serum and salivary α-2,3 sialoproteins was observed from controls to patients with OPC to oral cancer patients. The serum and salivary levels of α-2,3 sialoproteins were found to be significantly elevated in oral cancer patients as compared to the controls. The levels of serum and salivary α-2,3 sialoproteins were found to be higher in advanced stage of disease as compared to early stage of disease. Multivariate analysis showed significant association of serum α-2,6 sialoproteins with perineural invasion. The salivary levels of α-2,3 and α-2,6 sialoproteins were found to be decreased in CR as compared to PT levels while levels were comparable between PT and NR.

- An increasing trend of serum and salivary α-2,3 and α-2,6 ST was observed from controls to patients with OPC to oral cancer patients. The levels of salivary α-2,6 ST activity were found to be significantly higher in oral cancer patients as compared to controls. It was observed that the salivary levels of α-2,3 ST were found to be higher in patients with LN metastasis as compared to the patients without LN metastasis and were also higher in advanced stage of disease as

compared to early stage of disease. The levels of serum and salivary α-2,6 ST along with salivary α-2,3 ST were found to be significantly decreased in CR as compared to PT levels. The levels of serum α-2,3 ST, serum and salivary α-2,3 ST and α-2,6 ST were found to be increased in NR as compared to PT levels

- Serum α-2,6 sialoproteins were observed to be positively correlated with serum α-2,6 ST. Serum α-2,6 ST was observed to be significantly associated with α-2,3 ST. A significant positive correlation was observed between salivary α-2,6 ST and α-2,3 ST. Salivary TSA/TP ratio showed significant positive correlation with sialidase activity. Moreover, a significant negative correlation was observed between salivary α-2,6 ST activity and sialidase activity. Also salivary α-2,3 ST was negatively correlated with sialidase activity
- Fucoprotein analysis revealed an increasing trend of both serum and salivary 44 kDa fucoprotein from controls, to patients with OPC to oral cancer patients. Salivary fucoprotein levels were found to be significantly higher in oral cancer patients as compared to controls. The levels of serum and salivary 44 kDa fucoprotein were found to be higher in advanced stage of the disease as compared to early stage and levels were significant for saliva. Multivariate analysis showed significant association of salivary fucoproteins with stage of the disease. The analysis of serum and salivary fucoproteins in follow-up samples revealed decreased expression in CR compared to PT levels while levels were higher in NR as compared to PT levels.
- Serum and salivary α-L-fucosidase activity depicted an increasing trend from controls to patients with OPC to oral cancer patients. It was observed that serum and salivary α-L-fucosidase activities were higher in advanced stage of disease as compared to early stage and were also higher in patients with LN metastasis as compared to patients without LN metastasis. Serum α-L-fucosidase activity was observed to be significantly decreased in CR as compared to the corresponding PT value. Survival analysis showed that values above cut-off of serum α-L-fucosidase activity were significantly associated with lower overall survival.

- The results depicted that *ST3GAL1* mRNA expression was found to be higher in malignant tissues as compared to adjacent normal tissues. *FUT3* and *FUT5* transcripts levels were found to be significantly lower in malignant tissues as compared to the adjacent normal tissues. It was observed that mean levels of *ST3GAL1* and *FUT6* transcripts were found to be higher in metastatic tumors as compared to non-metastatic tumors. The levels of *FUT3* and *FUT5* were comparable between non-metastatic and metastatic tumors of the patients. It was observed that *ST3GAL1* and *FUT6* transcript levels were higher in advanced disease as compared to the early disease. *ST3GAL1* and *FUT5* transcripts levels were observed to be increased from well to moderate to poorly differentiated tumors. Kaplan Meir's survival analysis depicted significant lower survival in patients with expression above cut-off of *FUT3* transcripts in malignant tissues. Moreover, lower overall survival was observed for *FUT5* and *FUT6* transcripts with values above cut-off.

- The levels of plasma pro, active and total MMP-2 and MMP-9 were significantly higher in patients with OPC as compared to controls. Active MMP-2, pro and active MMP-9, total MMP-2 and MMP-9 were significantly higher in oral cancer patients as compared to controls. Active MMP-2 was observed to be significantly higher in oral cancer patients as compared to patients with OPC. The levels of plasma pro MMP-2, active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 were observed to be higher in advanced stage as compared to early stage of disease and levels were significant for pro MMP-2. Multivariate analysis depicted significant correlation of pro MMP-2, pro MMP-9, active MMP-9 and total MMP-9 with nuclear grade; active MMP-2 and total MMP-2 with lymphovascular permeation and pro MMP-2 with perineural invasion and infiltration. An increasing trend of plasma active MMP-2, pro MMP-9, active MMP-9, total MMP-2, total MMP-9 was observed from well to moderate to poorly differentiated tumors. Survival analysis depicted that the oral cancer patients with values above cut-off of plasma active MMP-2, pro MMP-9, total MMP-2 and total MMP-9 had lower overall survival as compared to those with values below cut-off.

- The expression of salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP were found to be significantly higher in oral cancer patients as compared to the controls, also the levels of pro MMP-9 and active MMP-9 were found to be significantly higher in oral cancer patients as compared to the patients with OPC. Activation ratio was found to be elevated in patients with OPC as well as in oral cancer patients as compared to the controls. The levels of salivary pro MMP-9, active MMP-9, truncated 42 kDa MMP and the activation ratio of MMP-9 were found to be higher in advanced disease and patients with LN metastasis as compared to early disease and patients without LN metastasis, respectively. Multivariate analysis depicted significant positive correlation of truncated 42 kDa MMP with infiltration.

- **ROC curve analysis** indicated that serum and salivary TSA/TP, serum and salivary sialidase activity, serum and salivary levels of α-2,3 sialoproteins, serum α-2,6 ST, salivary fucoprotein, serum and salivary α-L-fucosidase activity, plasma pro MMP-2, active MMP-2, pro MMP-9, total MMP-2 and total MMP-9, salivary pro MMP-9, active MMP-9 and truncated 42 kDa MMP, could significantly distinguish controls and oral cancer patients. Serum and salivary TSA/TP, serum sialidase, salivary α-2,3 sialoproteins, serum and salivary α-L-fucosidase activity, plasma pro MMP-2, active MMP-2, pro MMP-9, active MMP-9, total MMP-2 and total MMP-9 depicted significantly discriminating efficacy in distinguishing controls and patients with OPC. Moreover, Serum α-2,6 ST, salivary α-L-fucosidase activity, plasma pro MMP-2, pro MMP-9, active MMP-9, total MMP-9, salivary pro MMP-9 and active MMP-9 could significantly discriminate patients with OPC and oral cancer patients. Moreover, *FUT3* expression could significantly distinguish malignant and adjacent normal tissues.

- *ECAD* mRNA expression was found to be lower in infiltrating tumors as compared to non-infiltrating tumors. Multivariate analysis showed significant association between *ECAD* mRNA, tumor differentiation and stage of the disease. A decreasing trend of *ECAD* mRNA levels was observed from well to

moderate to poorly differentiated tumors. The levels were found to be significantly decreased in moderately differentiated tumors as compared to well differentiated tumors. Kaplan Meir's survival analysis of *ECAD* mRNA depicted lower survival with values below cut-off. The levels of truncated E-cadherin protein (97 kDa) were found to be higher in advanced stage of disease as compared to early stage of the disease. The levels of truncated E-cadherin protein were found to be increased in infiltrative tumors as compared to non-infiltrative tumors and were also significantly higher in metastatic tumors as compared to non-metastatic tumors. Multivariate analysis depicted significant association of truncated E-cadherin protein with metastasis.

- The levels of *CJUN* mRNA were comparable between paired malignant and adjacent normal tissues. *CJUN* mRNA expression was observed to be higher in early stage of the disease as compared to advanced stage of the disease. The levels of c-Jun protein were found to be significantly higher in malignant tissues as compared to adjacent normal tissues. c-Jun protein was found to be higher moderately differentiated tumors as compared to well differentiated tumors. c-Jun protein expression with values above cut-off depicted significant association with overall survival.

- It was observed that the levels of pEGFR expression were significantly higher in malignant tissues as compared to adjacent normal tissues. The levels were found to be higher in advanced stage as compared to early stage and were also found to be higher in metastatic tumors as compared to non-metastatic tumors.

Correlation between glycosylation, MMPs, pEGFR, c-Jun and E-cadherin: The result depicted significant positive correlation of serum α-2,6 sialoproteins with plasma pro MMP-2, active MMP-2, pro MMP-9, active MMP-9, total MMP-2, total MMP-9. Serum α-2,6 ST revealed significant positive correlation with plasma pro MMP-2, pro MMP-9 and total MMP-9. Serum α-L-fucosidase activity also showed significant positive correlation with plasma active MMP-2, pro MMP-9, active MMP-9, total MMP-2 and total MMP-9. The correlation analysis between salivary MMPs and salivary glycosylation changes exhibited that salivary α-2,6 sialoproteins were

positively correlated with pro MMP-9, active MMP-9 and truncated 42 kDa MMP. Salivary α-2,3 sialoproteins showed significant positive correlation with truncated 42 kDa MMP and salivary sialidase activity with active MMP-9 and truncated 42 kDa MMP. The results summarised that increased glycosylation/alterations of glycoproteins is associated with elevated levels of MMPs.

The present study depicted a significant positive correlation between truncated E-cadherin protein and *CJUN* mRNA. Moreover, *ECAD* mRNA was observed to be positively correlated with *CJUN* mRNA.

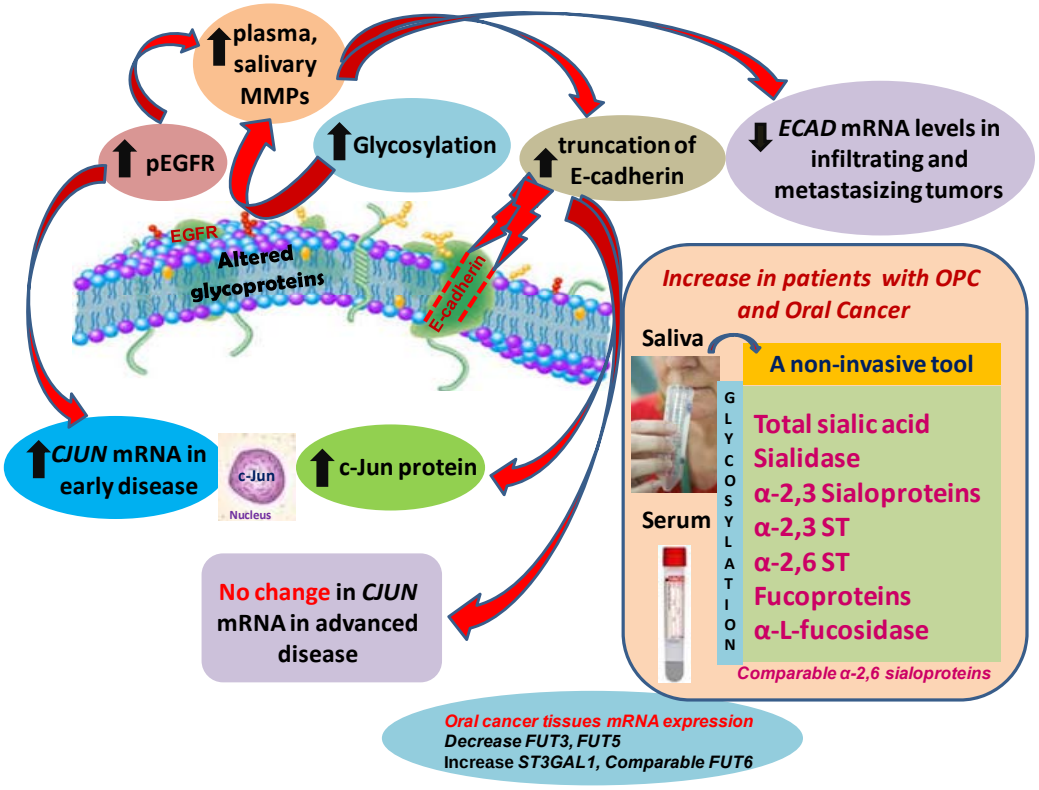

Figure 7.1: ***Schematic representation of the study outcome***

Truncated E-cadherin protein was observed to be positively correlated with c-Jun protein. Moreover, truncated E-cadherin protein showed significant positive correlation with plasma pro MMP-2, plasma active MMP-2, plasma active MMP-9, plasma total MMP-2 and activation ratio of MMP-2. Thus, increased MMPs might be

involved in upregulation of truncated E-cadherin protein and decreased *ECAD* mRNA levels in infiltrating and metastasizing tumors **(Figure 7.1)**.

pEGFR expression and *CJUN* mRNA transcripts exhibited significant positive correlation. *CJUN* mRNA levels were observed to be higher in early stage as compared to advanced stage. Hence, we summarise that during early stage of the disease, EGFR–JNK/ERK pathway might be involved in increased c-Jun protein as well as mRNA levels **(Figure 7.1)**. During advanced stage of the disease, the increase in c-Jun protein might be accomplished by IRES mediated translation due to loss of E-cadherin protein. **Figure 7.1** is the schematic representation of the study outcome, which describes comprehensively the summary of results of the present study.

CONCLUSION:

- The alterations in serum glycoprotein electrophoretic pattern observed in patients with OPC and oral cancer patients might aid in early detection of oral cancer. Increased serum/salivary glycosylation observed in patients with OPC and oral cancer patients, reflect the usefulness of serum as well as saliva (a non-invasive tool) in early detection and disease monitoring, which might be ideally used for early screening programmes in clinical set-up.
- A significant increase in plasma active MMP-2, total MMP-2, Pro MMP-9, active MMP-9 and total MMP-9 observed in patients with OPC and oral cancer patients as compared to the controls, reflects its usefulness in early detection of metastasis. The increased salivary levels of pro MMP-9, active MMP-9, and truncated 42 kDa MMP in oral cancer patients as compared to the controls highlight the importance of non-invasive tool to predict metastasis in oral cancer patients.
- Decrease in *ECAD* mRNA expression and an increase in 97 kDa truncated E-cadherin in infiltrating tumors as compared to non-infiltrating tumors and association of *ECAD* mRNA with differentiation and stage of the disease, documents loss of E-cadherin and restructuring of cytoskeleton during metastatic and advanced stage of disease.

- The increase in glycosylation causes alterations in membrane glycoproteins and thus there is disruption of cell-cell adhesion due to increase in protease activity of MMP-2 and MMP-9. No change in *CJUN* mRNA levels reflects that increase in c-Jun protein observed in malignant oral cancer tissues might be due to restructuring of cytoskeleton due to loss of E-cadherin by MMPs. This represents novel pathway for upregulation of c-Jun protein, an important drug target, which has not been studied earlier in oral cancer patients. The increase in *CJUN* mRNA in early stage of disease as compared to advanced stage of disease and a positive correlation with pEGFR depicts that increase in c-Jun protein in early stage of disease might be due to MAPK pathway. This represents differential pathway of c-Jun protein expression during early and advanced stage of disease.
- The data also suggested that glycosylation along with other molecular events like EGFR, MMPs and c-Jun might serve to identify newer drug targets for oral cancer.

FUTURE SCOPE:

In furtherance, characterization of altered glycoproteins by glycoprotein enrichment strategies and Mass spectrometry analysis might give insights into specific glycoproteins involved in oral carcinogenesis. Purification of glycosyl moieties from glycoprotein can give insights into different carbohydrate residues involved in altered glycoprotein expression in oral carcinogenesis. Evaluation of other transcripts subtypes of ST and FUT might give insights into involvement of specific subtypes in oral cancer pathogenesis.

Additional studies on drugs targeting sialylation and fucosylation alone or in combination with EGFR, MMPs and c-Jun can be further examined in oral cancer patients. Assessment of different intermediates of pathway of c-Jun expression like JNK/ ERK along with other cytoskeleton markers of EMT can be evaluated to understand the detailed mechanism of c-Jun overexpression in oral cancer. Further assessment of parameters with a larger sample size might be helpful in evaluating its efficacy in clinical set-up for maximum benefits of oral cancer patients.