4. Results and discussion

4.1 Study the role of TNF-α -regulated mitochondrial proteins in ER/PR (+ve) and ER/PR (-ve) breast cancer cells.

4.1.1 Proteomic analysis of mitochondrial proteins in ER/PR +ve and -ve breast cancer cells in presence and absence of TNF-α

To identify the role of TNF-α in regulating mitochondrial proteins in MCF-7 (ER/PR +ve) and MDA-MB-231 (ER/PR –ve) cells, we performed quantitative proteomic analysis of mitochondrial proteins of both cell lines in the presence and absence of TNF-α. Mitochondrial fractions were prepared from MCF-7 and MDA-MB-231 cells and the purity was assessed by western blotting using selected mitochondrial marker proteins (Tom20, UQCRC2, and SDHA) including nuclei (PARP), cytosol (β-actin) (Figure 6A). The mitochondrial fraction of both MCF-7 and MDA-MB-231 showed high levels of mitochondrial proteins of TOM20, UQCRC2, and SDHA whereas PARP, a marker of nuclei, was not detected. Ingenuity pathway analysis (IPA) indicated that DEPs were involved in mitochondrial function (20%), Sirtuin Signaling Pathway (18%), oxidative phosphorylation (12%), protein ubiquitination (12%), and NRF2-mediated oxidative stress (10%) in ER/PR +ve (MCF-7) cells. Interestingly, IPA analysis of DEPs in ER/PR -ve (MDA-MB-231) cell line showed that proteins involved in mitochondrial dysfunction, oxidative phosphorylation, sirtuin signaling, and fatty acid β-oxidation were enriched in presence of TNF-α as compared to MCF-7 cell lines (Figure 6B).

LC-MS/MS data of MCF-7 +/- TNF-α and MDA-MB-231 +/- TNF-α were searched against the decoy UniProt human database and identified 1,077 and 1,150 mitochondrial proteins (Peptide probability >95%; Protein probability >99%) for MCF-7 and MDA-MB-231 cells, respectively. The list of identified mitochondrial proteins were further compared with mitochondrial protein databases; MitoCarta and Gene Ontology. It was observed that 57.5% of proteins overlapped between MDA-MB-231 and MCF-7 cells (Figure 6C). The results here suggest that the TNF-α differentially modulates cellular pathways and mitochondrial proteome in both ER/PR +ve (MCF-7) and ER/PR –ve (MDA-MB-231) cells.

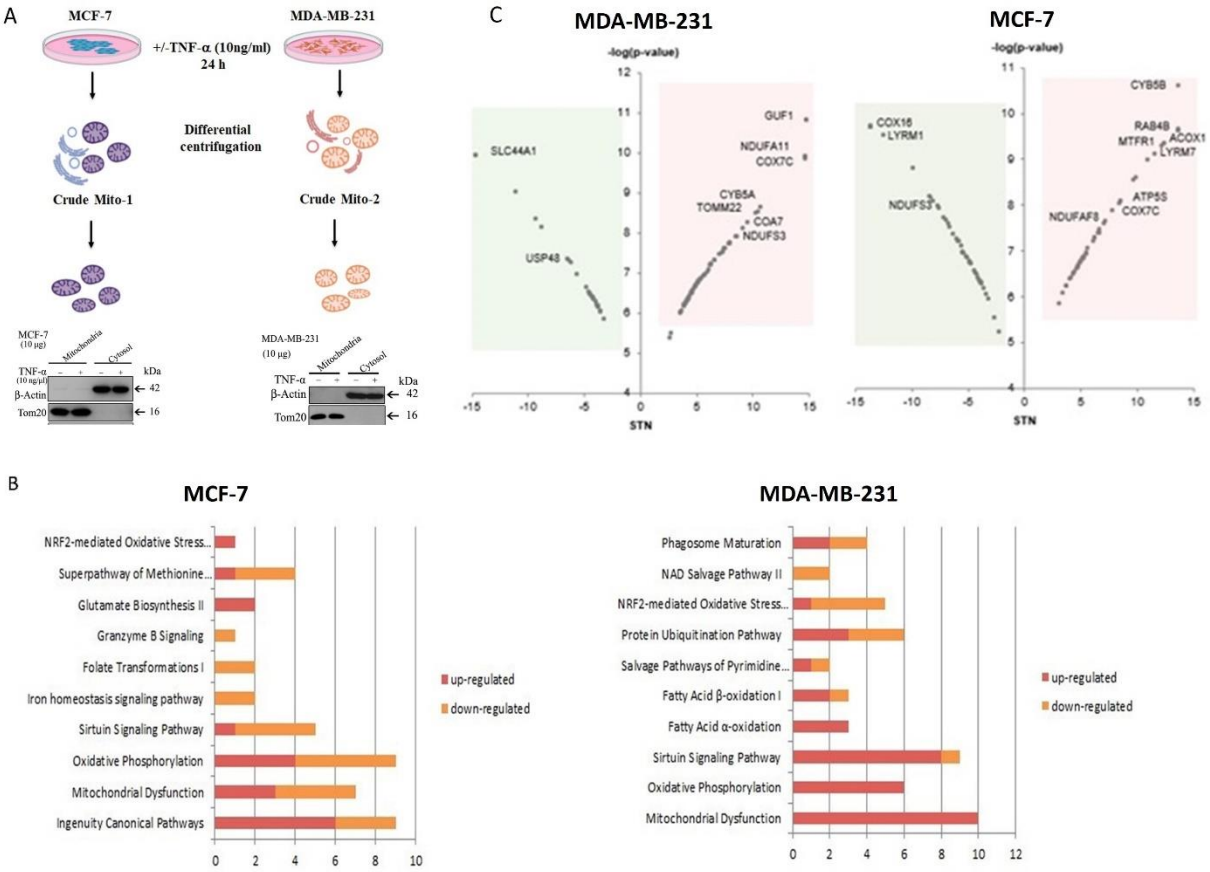


Figure 6: Quantitative proteomic analysis of TNF-α regulated mitochondrial proteins in breast cancer cells. (A) Immunoblot analysis of isolated mitochondria (B)Molecular functions of DEPs by IPA tool. The x-axis indicates the number of proteins, red bar as up-regulated and orange bar as down-regulated proteins. (C)Volcano plots of DEPs displayed in the P-value (−log10) versus signal-to-noise ratio (STN). The p-value and STN were determined by power law global error model (PLGEM) statistical analysis for label-free quantification

4.1.2 Analysis of TNF-α induced differential expression of mitochondrial proteins involved in assembly and functions of mitochondrial complexes

The remodeling of electron transport chain (ETC) complexes assembly and/or activity is important for the bioenergetic adaptation in cancer cells [230], hence we focused specifically on the individual protein constituents of each respiratory complex. All the known subunits of ETC complexes including complex I, II, III, IV were analysed both in MCF-7 and MDA-MB-231 in presence of TNF-α. The levels of NDUFS3 (a N module component) and NDUFB1 (the component of ND4 module) significantly decreased in MCF-7 cells in the presence of TNF-α however, increased in MDA-MB-231 (Figure 7A). The levels of NDUFA11, the matrix-facing subunit of CI increased several folds in MDA-MB-231 cells and decreased in MCF-7 in the presence of TNF-α. This suggests that the levels of mitochondrial complex I proteins are differentially altered in ER/PR +ve (MCF-7) and ER/PR –ve (MDA-MB-231) cells in presence of TNF-α.

Complex-II is the smallest mitochondrial complex and unique as it forms a part of the TCA cycle as well as a part of ETC hence directly linking metabolism and oxidative phosphorylation [231]. Therefore, the study further analysed the levels of complex-II subunits in MCF-7 and MDA-MB-231 cells in presence of TNF-α. MCF-7 showed increased levels of SDHD subunit whereas other subunits: SDHA and the assembly factor, SDHA11 decreased in presence of TNF-α (Figure 7B). MDA-MB-231 cells treated with TNF-α showed no change in SDHD level whereas the level of SDHA and SDHB increased in mitochondria. The level of SDHA11 was significantly high in mitochondria of TNF-α treated MDA-MB-231 cells as compared to MCF-7.

Mitochondrial complex-III is an important complex as it accepts electrons both from complex-I and complex-II via the acceptor, ubiquinone. The alteration of complex-III may lead to oxidative stress and accumulation of oncometabolite leading to increased cell proliferation. We further analysed the level of Complex-III subunits from the mitochondrial proteome of both MCF-7 and MDA-MB-231 cells in presence of TNF-α. Levels of most subunits of complex-III remained same both in MCF-7 and MDA-MB-231 in presence of TNF-α. Interestingly, LYRM7, a protein for complex-III assembly showed altered levels in both cell lines. The level of LYRM7 significantly increased in MCF-7 in presence of TNF-α as compared to MDA-MB-231 (Figure 7C). To confirm this, the expression levels of LYRM7 levels were also analysed by western blotting. LYRM7 protein levels also increased in mitochondrial fraction of TNF-α treated MCF-7 cells as compared to MDA-MB-231 cells (Figure 7E). UQCRC2, a complex-III subunit also decreased in TNF-α treated MDA-MB-231 mitochondrial fraction and correlated with proteomics data (Figure 7E). Similarly, BCS1L, a 419-amino-acid chaperone protein, is a member of the family called AAA, is localized in the inner membrane of the mitochondria, and presumed to facilitate the insertion of Rieske Fe/S protein into precursors to complex-III [232]. The study also analysed the level BCS1L in mitochondrial proteome and observed increased levels in mitochondria of MDA-MB-231 cells in presence of TNF-α whereas remained unchanged in MCF-7 cells.

Complex-IV subunit levels were also analysed in mitochondrial fraction of both MCF-7 and MDA-MB-231. No significant changes were observed in the levels of different cytochrome-c oxidase (COX) complex subunits. However, interestingly the assembly factors required for the complex-IV biogenesis were differentially regulated in presence of TNF-α in the breast cancer cells. COX14 assembly factor plays an important role in the translation of the COX1, the main constituent of complex-IV [5][233]. The levels of COX14 decreased significantly in the mitochondria of MDA-MB-231 as compared to MCF-7 in the presence of TNF-α (Figure 7D). Similarly, another assembly factor, COX16 was downregulated in MCF-7 cells as compared to MDA-MB-231.

Altogether, the above evidence from quantitative mitochondrial proteomics and immunoblotting suggests that protein levels of specific subunits of mitochondrial ETC complexes are differentially regulated which may, in turn, modulate the assembly and/or activity in presence of TNF-α.

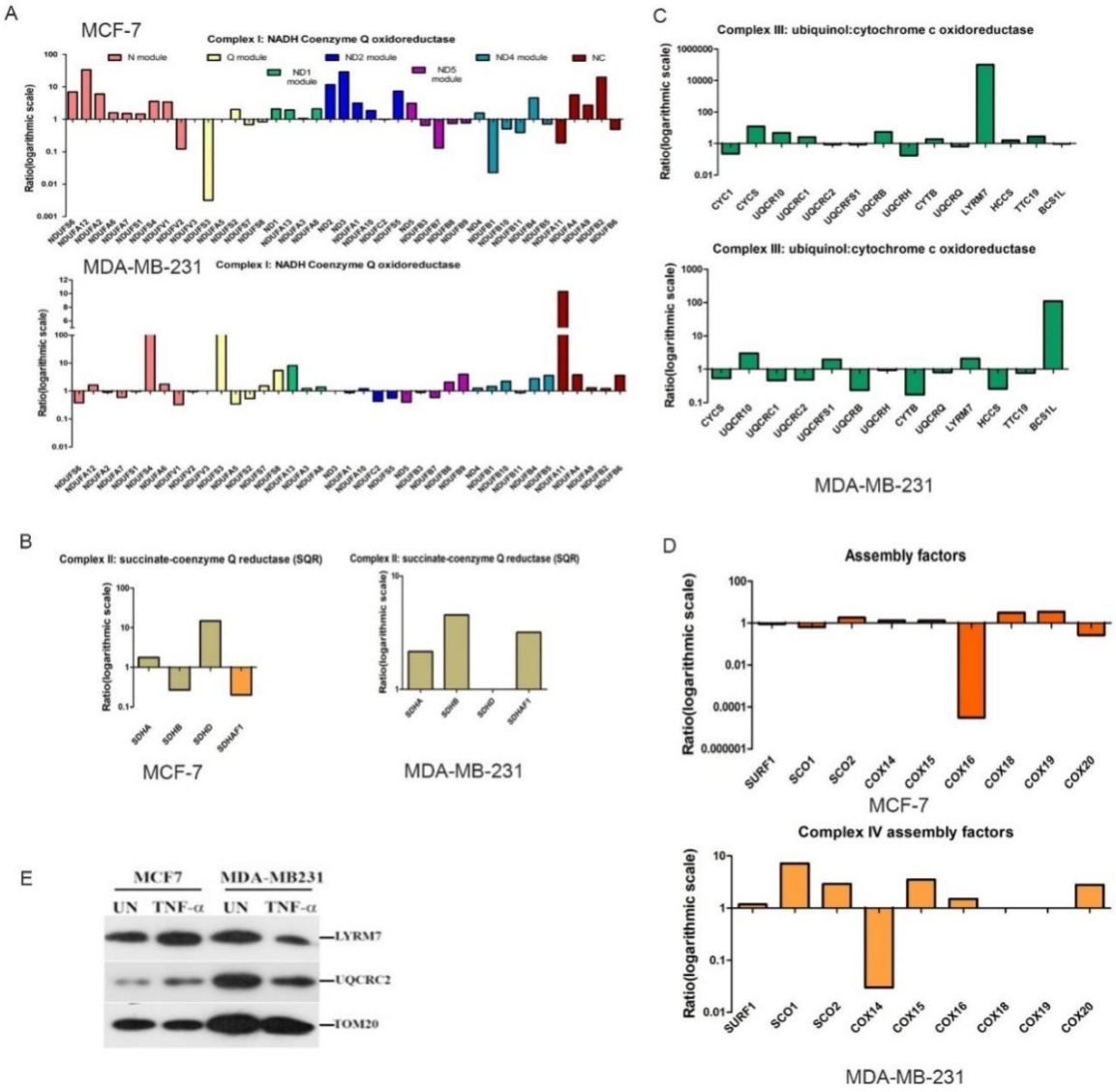


Figure 7: TNF-α regulated mitochondrial proteins in breast cancer cells. *The raw protein abundance count of mitochondrial protein subunits from untreated and TNF-α treated triplicates from both cells were exported and plotted as a ratio of log-fold change. The encircled proteins show the log-fold change of unique proteins in both cells.* ***(A)*** *The protein subunits of the CI complex are clustered and color-coded into respective modules according to their function. NC represents a non-characterized component of CI complex.* ***(B)****,* ***(C)&(D)*** *The log-fold change of unique proteins for CII, CIII & CIV in both cell lines respectively* ***(E)*** *western blot analysis of complex III proteins UQCRC2 and LYRM7 in presence MCF-7 and MDA-MB-231 in presence of TNF-α*

4.1.3 Discussion:

An increased level of cytokines in TME of solid tumors may modulate mitochondrial function for metabolic adaptation however its systemic role in the regulation of mitochondrial proteome and OXPHOS assembly is not well understood. The regulation of TNF-α mediated mitochondrial complex assembly and its role in the regulation of tumorigenic potential of different breast cancer cell types had not been systemically investigated. TNF-α is known to activate many transcriptional factor including members of NF-κB family which may regulate the expression of genes involved in different steps of metabolic adaptation [234]. To understand the differential regulation of TNF-α on mitochondrial proteome, the study systemically characterized mitochondrial proteins of two different cell types MCF-7: ER/PR +ve, responsive representing early tumor conditions, and MDA-MB-231 cells: ER/PR –ve representing aggressive and metastatic conditions tumor cell types. TNBC cells have been well characterized for their metabolic alterations that include decreased mitochondrial respiration [235]. The mechanism behind such alterations is still unknown and needs to be understood.

There is no systemic study monitoring the mitochondrial proteome in different breast cancer subtypes which determines differential metabolic adaptations in TME. Recent studies have targeted ETC in mitochondria with inhibitors like hypericin photodynamic therapy (HYP-PDT) [236]. A combination of Tamoxifen and HYP-PDT was tested in metabolically different MCF-7 and MDA-MB-231 and this resulted in 90% cytotoxicity in both the cell lines. Such studies highlight the need of combinational therapies targeting components of TME along with mitochondrial functions. Hence it is important to understand the TNF-α modulated mitochondrial proteome to understand the differential mitochondrial role in driving the tumor characteristic and heterogeneity.

The high-resolution proteomics clearly showed that TNF-α differentially modulates mitochondrial proteome of MDA-MB-231 and MCF-7 cells that may lead to defects in mitochondrial functions and complex assembly. Other pathways that are differentially regulated in presence of TNF-α like sirtuin pathways and iron homeostasis in MCF-7 and MDA-MB-231 cells, also regulate mitochondrial functions however this can be a topic of further investigation. The level of mitochondrial DNA encoded transcripts specifically ND2 and ND3 which form part of the core unit of the complex-I, increased in MCF-7 whereas remaining unaltered in MDA-MB-231 cells. The levels of NDUFS3 (N module component) and NDUFB1 (the component of ND4 module) was significantly decreased in MCF-7 cells in

the presence of TNF-α however, increased in MDA-MB-231 suggesting that TNF-α can differentially modulate the complex-I activity. NDUFS3 was identified as a potential biomarker of breast cancer aggressiveness [237]. Reports have identified distinct differences in mitochondria of normal and breast infiltrating ductal carcinoma (IDC) cells. A significant decrease was observed in the levels of SDHB, NDUFS3 and UQCRC2 in the mitochondria of IDC compared to normal cells [238].

Complex II, is the only complex that participates in OXPHOS as well as TCA cycle. Our study observed a significant decrease in the SDHB and SDHAF1 which are major proteins for complex II in MCF-7 compared to MDA-MB-231. It was observed that impaired complex II activity by DT-010 showed decreased mitochondrial membrane potential and ATP production which further led to reduced proliferation in MCF-7 and MDA-MB-231 cells [239]. Such studies show that selectively targeting mitochondrial complexes may have anti-cancer effects and needs to be explored.

Similarly, mitochondrial complex-III, which can accept electrons from both complex-I and complex-II through ubiquinone is critical for mitochondrial function. Our study identified LYRM7, an essential assembly factor to be increased in MCF-7 as compared to MDA-MB-231. Recent study targeting lactate metabolism in breast cancer identified LYRM7 as a critical gene associated with high immune infiltration in BC [240]. LYRM7 was also identified as a target gene of miR-24-2 in TNBC [241]. The survival of BC patients was associated with low expression of LYRM7 in this study.

Further, complex IV subunits like COX14 and COX16 showed differential expression in presence of TNF-α in MCF-7 and MDA-MB-231 cells. Mutations in structural genes of complex IV were identified in 2107 breast cancer patient samples and were highlighted as potential targets for breast cancer [242].

Studies have shown that mitochondrial complexes and ETC functions as critical regulators of cancer biology [243, 244]. The study here suggests that many critical proteins of different complexes of OXPHOS are differentially regulated by TNF-α in ER/PR positive and ER/PR negative cell type. It will be important to explore their functional implication if OXPHOS activity and tumorigenic potential. Moreover, such differential mitochondrial protein expression in presence of inflammation are crucial targets for studying their effects in breast cancer cells survival and proliferation.

4.2 Study the role of TNF-α-regulated mitochondrial functions and metabolism in ER (+ve) and ER (-ve) breast cancer cells and determining their migration and clonogenic ability.

4.2.1 TNF-α differentially regulates mitochondrial supercomplex assembly and activity in ER/PR +ve (MCF-7) and ER/PR –ve (MDA-MB-231) breast cancer cells

To understand the implication of TNF-α modulated subunits of mitochondrial ETC complexes, the organization and activity of ETC complexes were analysed from both MCF-7 and MDA-MB-231 cells in the presence/absence of TNF-α using Blue native-PAGE. The study observed that TNF-α decreased the levels, as well as the activity of supercomplex (SC) containing Complex-I and Complex-IV in both the cell lines, however, this decrease was significantly higher in MDA-MB-231 cells in the presence of TNF-α (Figure 8A). A significant decrease in individual complex-III activity was observed in MDA-MB-231 in presence of TNF-α compared to MCF-7 (Figure 8B).To further quantify activity of individual ETC complexes, the specific activity of complex-I and complex-II using the spectrophotometric assay were monitored in MCF-7 and MDA-MB-231 cells in the presence of TNF-α either alone or combination with 2-deoxyglucose (2-DG). 2-DG, the glucose analog, inhibits glycolysis. Complex-I activity significantly decreased in presence of TNF-α in MDA-MB-231 as compared to MCF-7 cells. The inhibition of glycolysis with either 2DG alone or in combination with TNF-α increased complex-I activity in MCF-7 cells. On the other hand, the activity of complex-I decreased significantly upon inhibition of glycolysis by 2-DG in MDA-MB-231 but remained unchanged in presence of both 2-DG and TNF-α (Figure 8C). These results suggest that TNF-α negatively regulates complex-I activity in MDA-MB-231 cells which depends on glycolysis to maintain its activity in contrast to MCF-7.

The analysis of complex-II activity revealed no significant changes either in MCF-7 or MDA-MB-231 in the presence of TNF-α (Figure 8D). However, treatment with 2-DG alone or in combination with TNF-α significantly increased complex-II activity in MDA-MB-231 suggesting a compensatory response by complex-II to maintain the overall ETC function during glycolytic inhibition. Altogether, these results indicate that TNF-α modulates the levels of critical components of complex-I, complex-III, and complex-IV, hence differentially regulate the organization and activity of ETC complexes in MDA-MB-231 cells (ER/PR –ve) and MCF-7 cells (ER/PR +ve) in presence of TNF-α.

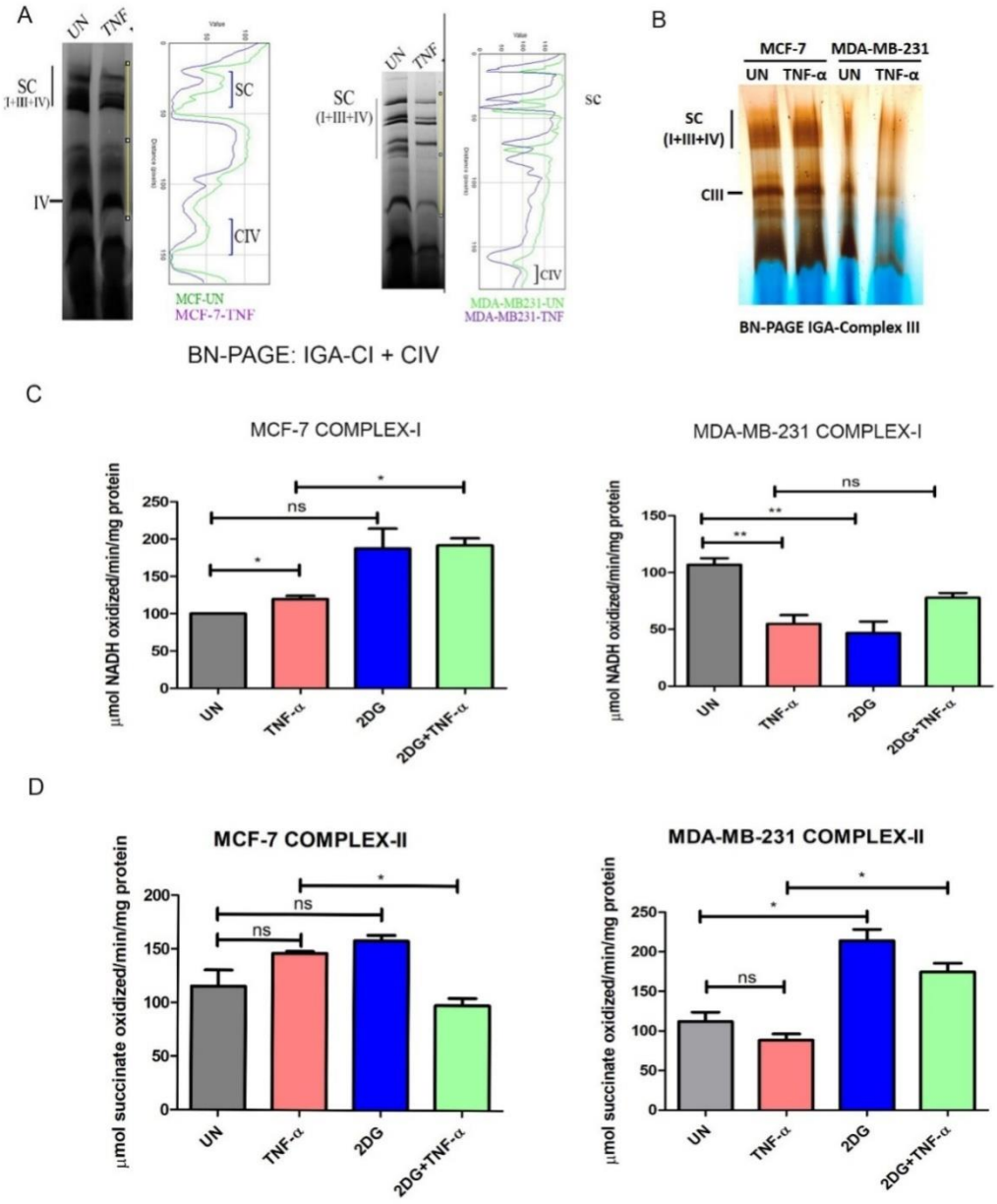


Figure 8: TNF-α alters the OXPHOS assembly and activity in breast cancer cells. (A) MCF-7 and MDA-MB-231 cells were treated with TNF-α (10 ng/µl) for 24 h. After treatment, the mitochondrial fraction was isolated and further analysed by BN-PAGE, and in-gel enzyme staining for CI and CIV was performed as described in Methodology. **(B)**Complex III in-gel activity was analysed by BN-PAGE and enzyme staining as described in methodology (**C**) & (**D**) MCF-7 and MDA-MB-231 cells were treated with TNF-α (10 ng/µl) and 2-DG (10 mM) either alone or in combination for 24 h and Complex-I and Complex-II activity was measured spectrophotometrically as described in Methodology.

4.2.2 TNF-α downregulates ATP levels and enhances ROS generation in triple-negative MDA-MB-231 cells

To investigate the effect of TNF-α regulated ETC complexes assembly/activity on the mitochondrial bioenergetic status of both the cells, the level of ATP and ROS in the presence/absence of TNF-α was analyzed. In accordance with the above results, TNF-α treatment significantly decreased both total cellular and mitochondrial ATP level in MDA-MB-231 cells but not in MCF-7 cells. MCF-7 cells cultured in presence of 2-DG in the presence/absence of TNF-α showed a significant decrease in total ATP levels however the mitochondrial ATP levels increased significantly under all treatment conditions suggesting an upregulated ETC function (Figure 9A and 9B). In contrast, MDA-MB-231 cells displayed increased sensitivity to a decrease in both mitochondrial and total cellular ATP levels in the presence of TNF-α with or without 2-DG. This result suggested that MDA-MB-231 cells are strongly dependent on the glucose-pyruvate axis for substrate oxidation and ATP generation by OXPHOS, which is altered in presence of TNF-α.

Similarly, both intracellular and mitochondrial ROS levels significantly increased in both cell lines however MDA-MB-231 cells displayed an enhanced ROS generation in the presence of TNF-α (Figure 9C) as compared to MCF-7. Altogether, these results strongly suggest that TNF-α alters the mitochondrial bioenergetic status of MDA-MB-231 cells by negatively regulating the ETC complexes activity leading to a decrease in ATP levels and increased ROS generation.

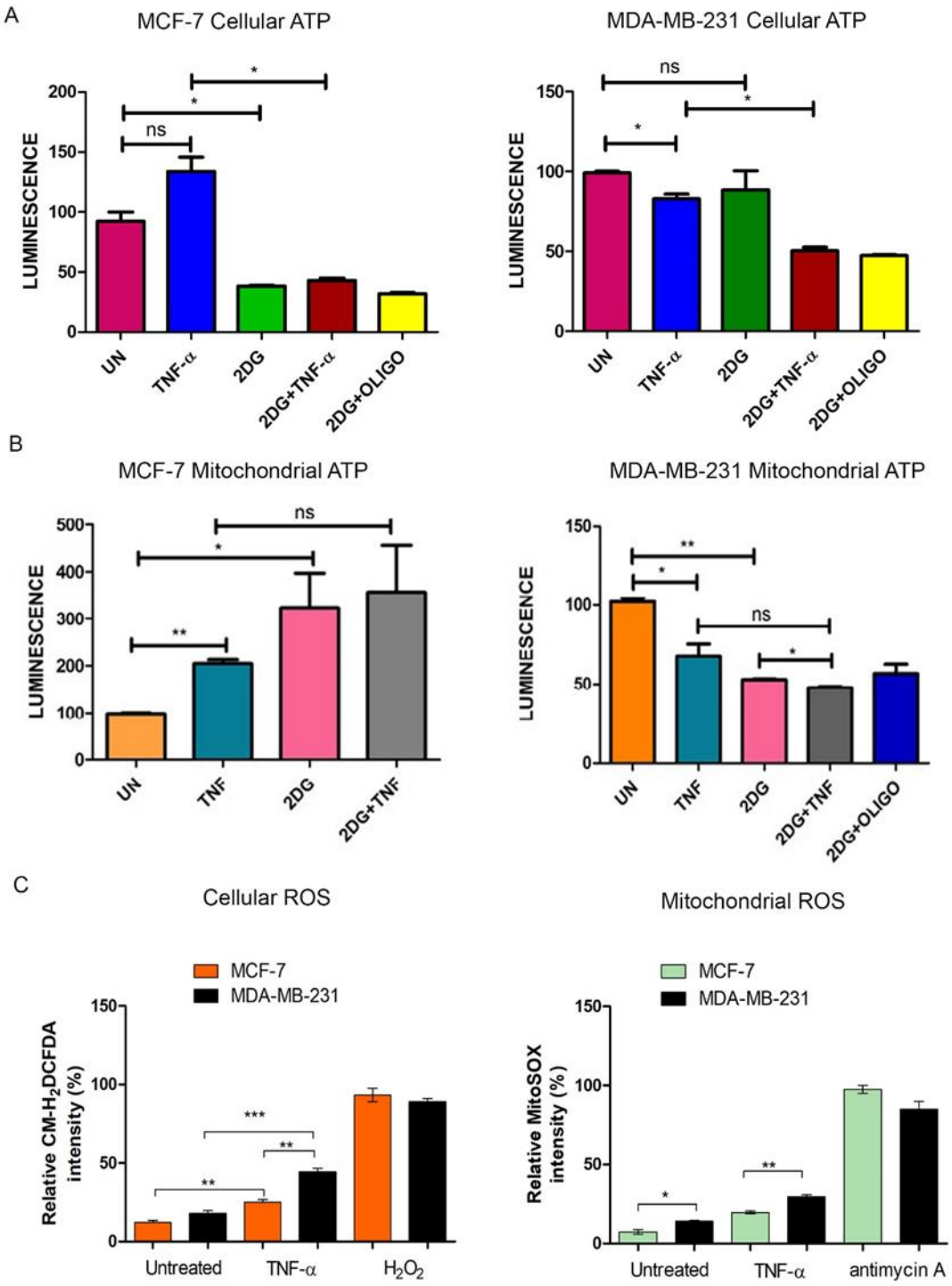
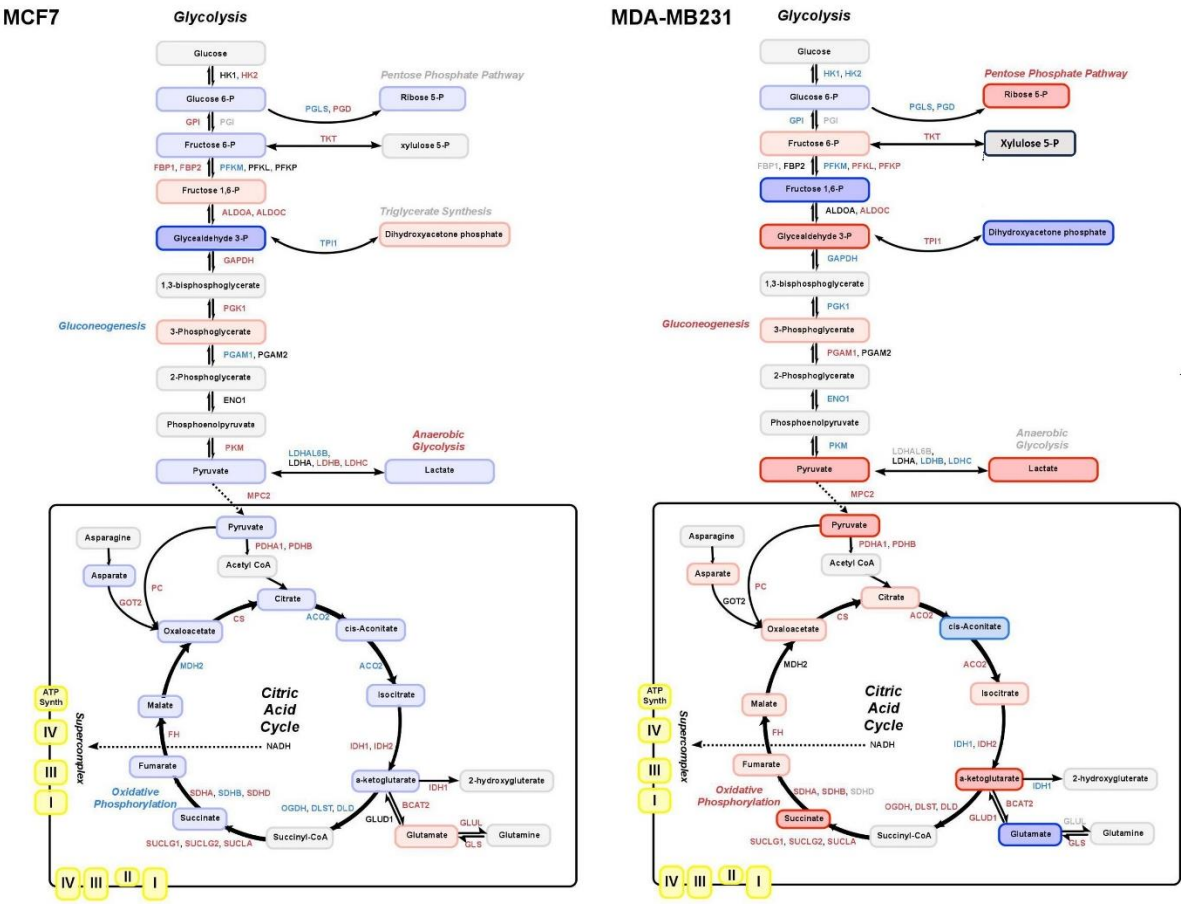


Figure 9: TNF-α decreases ATP levels and increases ROS generation in MDA-MB-231 cells. (A)MCF-7 and MDA-MB-231 cells were treated with TNF-α wither alone or in combination with 2-DG (10 mM) and the total steady-state ATP levels were determined by the luciferase-based assay as described in Methodology. **(B)** As in (A), mitochondria from respective cells were isolated upon treatment and ATP levels were measured. Oligomycin

(5 µg) treatment for 20 min was used as a positive control. **(C)** MCF-7 and MDA-MB-231 cells were treated with TNF-α. After treatment, the cells were stationed with fluorogenic ROS-sensitive dye and relative fluorescence was monitored as described in Methodology. H_2O_2 (100 µM) and antimycin A (10 µg) treatment for 2 h were used as a positive control.

4.2.3 Targeted metabolomics analysis showed TNF-α differentially regulate metabolic pathways in ER/PR +ve and ER/PR-ve breast cancer cells

Mitochondrial ETC complexes regulates NAD/NADH ratio, hence regulates metabolic adaption during different stress conditions. TNF-α mediated differential regulation of metabolism in MCF-7 (ER/PR +ve cell line, luminal), and MDA-MB-231 (ER/PR -ve cell line, basal) is not well understood. It is observed that glycolysis and TCA cycle is not altered in MCF-7 in presence of TNF-α (Figure 10). The increased accumulation of fructose6-phosphate and 3-phosphoglycerate was observed in MDA-MB-231 suggesting that glycolysis is reprogrammed for the nucleotide biosynthesis in the presence of TNF-α for survival. Further, these metabolites are diverted to pentose phosphate pathway for the nucleotide biosynthesis as increased level of ribose-5 phosphate is observed in MDA-MB-231 cells as compared to MCF-7 cells. The electron acceptors like pyruvate and aspartate are high in presence of TNF-α in MDA-MB-231 cells as compared to MCF-7. The level of α-ketoglutarate was high in MDA-MB-231 which is precursor of D-2-hydroxyglutarate (D2-HG), an oncometabolite. The level of succinate, another oncometabolite was also high in MDA-MB-231 cells in presence of TNF-α, which correlates with decreased level of SDH subunits. The level of glutamine in MDA-MB-231 cells and its derivative decreased in MDA-MB-231 cells suggesting its increased flux in presence of TNF-α, whereas it was low in MCF-7 cells. These experiments suggest that TNF-α differentially regulates metabolic adaption of breast cancer cells subtypes.



Metabolite	Area MCF7 TNF (-)	Area MCF7 TNF (+)	Log2FC	Area MB231 TNF (-)	Area MB231 TNF (+)	Log2FC
Glucose6P	0.0912	0.0857	-0.0823	0.0657	0.0597	-0.1212
R5P	1.0167	0.7656	0.0219	0.7601	1.1731	0.5942
Fructose6P	0.5470	0.4470	-0.2545	0.3399	0.4043	0.2569
Frutose1,6P	0.2146	0.2191	0.0345	0.5238	0.3351	-0.6055
glyceraldehyde3p	0.1310	0.0813	-0.6684	0.0249	0.0622	1.3257
dihydroxyacetone phosphate	0.0892	0.0941	0.0842	0.6358	0.3913	-0.6990
3phosphoglycerate	0.1790	0.2107	0.2549	0.0935	0.1239	0.4065
Pyruvate	0.7071	0.6081	-0.1902	0.3949	0.6298	0.6745
Lactate	249.5234	203.9906	-0.2411	126.8107	198.2838	0.6470
Citrate	319.5226	243.9318	-0.3725	165.3576	186.6718	0.1764
Aconitate	2.2196	1.5748	-0.4959	1.3431	1.4845	0.1343
iso-Citrate	1.4355	1.0492	-0.4556	0.9271	0.9948	0.1054
aKetoglutarate	7.4300	5.8554	-0.3472	4.5159	5.7001	0.3329
Glutamate	131.9145	171.2360	0.4038	185.0457	130.9984	-0.4957
Succinate	93.0360	77.4311	-0.2450	28.4548	59.8769	1.0737
Fumarate	1.3202	1.0195	-0.3787	0.9576	1.2521	0.3976
Malate	79.8082	61.3697	-0.3821	57.6848	77.0514	0.4217
Oxaloacetate	2.9086	2.6397	-0.1383	2.0024	2.2050	0.1360
Aspartate	28.4151	26.8727	-0.0463	3.5456	3.3735	0.0339

Figure 10: Metabolites levels in presence of TNF-α. The abundance of metabolites in presence and absence of TNF-α in MCF-7 and MDA-MB-231.

4.2.4 TNF-α modulated mitochondrial functions differentially regulate migration and clonogenic ability of breast cancer cells

To further investigate the role of TNF-α modulated mitochondrial OXPHOS complex activity in regulating migration and tumorigenic potential of ER/PR +ve: MCF-7 and ER/PR-ve: MDA-MB-231 cells, the migration ability of both cells was checked in presence of TNF-α and observed that TNF-α enhanced the migration ability of MDA-MB-231 cells as compared to MCF-7(Figure 11A). Further, the study analysed the clonogenic ability in the presence/absence of TNF-α. Interestingly, TNF-α inhibits the clonogenic ability of MCF-7 cells whereas enhances clonogenicity of MDA-MB-231 cells (Figure 11B). The addition of 2DG in presence of TNF-α further reduced clonogenic ability of the MDA-MB-231 cells suggesting the glycolysis is essential for MDA-MB-231 cells. The rescue of clonogenicity was observed in presence of NAC (N-Acetyl cysteine), a ROS scavenger in MDA-MB-231 cells whereas it was not observed in the MCF-7 cells.

Previously it had been observed that electron acceptors are limited to drive ETC and other anaplerotic reactions in cancer cells (TCA in cancer). A significant accumulation of pyruvate was observed in metabolites study in presence of TNF-α in MDA-MB-231 as compared to MCF-7. Hence, the clonogenic ability of these cells was monitored in presence of pyruvate. An increase in the number of colony-forming units in culture medium supplemented with pyruvate was observed in both the cells. This suggests that electron acceptor maintains the ratio of NAD/NADH in the cell to drive the TCA cycle and glycolysis in MCF-7 whereas pyruvate, the electron acceptor is not limiting factor. The presence of pyruvate can rescue clonogenic ability in presence of TNF-α in MCF-7 (Figure 11C). Interestingly there was no major change in pyruvate stimulated clonogenic ability of MDA-MB-231 cells in presence of TNF-α.

Hemin is known to degrade BTB and CNC homology1 (BACH1), a haem binding transcription factor that is increased in TNBC tumors and enhance mitochondrial respiratory activity. To further confirm, the reliance of MDA-MB-231 on OXPHOS activity, cell viability of MDA-MB-231 was checked in presence of TNF-α in combination with Hemin. The cell viability of MDA-MB-231 cells was observed to be significantly decreased in presence of TNF-α and Hemin (Figure 11D). This suggests that enhancing the mitochondrial proteins and functions in MDA-MB-231 cells in presence of TNF-α can inhibit the triple-negative breast cancer cell survival.

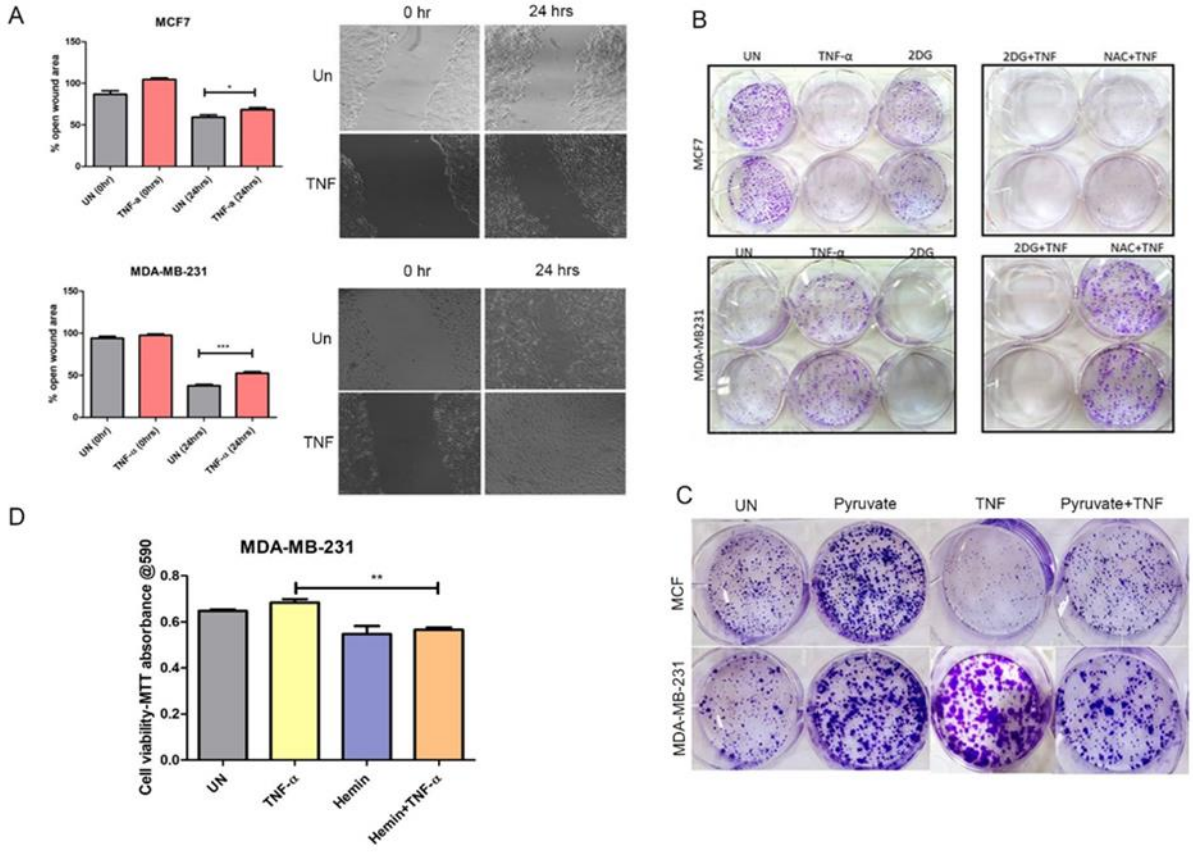


Figure 11: TNF-α sensitizes cell death and decreases growth in MDA-MB-231 cells (**A**) MCF-7 and MDA-MB-231 cells were seeded in 12 well plates &wound scratch assay was performed in presence of TNF-α (**B**)&(**C**) MCF-7 and MDA-MB-231 cells were seeded in low number as described in methodology and their ability to form colonies in the presence and absence of TNF-α,2-DG, NAC and pyruvate was monitored (**D**) MDA-MB-231 cells were treated with TNF-α either alone or in combination with Hemin (10 mM) and the cell viability was assessed as described in Methodology by MTT.

4.2.5 Subunit of mitochondrial complexes negatively correlates with survival of the breast cancer patients

The TIMER database is a web resource used for systemic analysis and evaluation of clinical impacts of different immune factors in diverse cancer types hence the study analysed the correlation between TNF-α and the expression level of identified DEPs. The TIMER data showed that increased expression of TNF-α in basal breast cancer patients is associated with decreased gene expression of subunits of mitochondrial Complex-I. This negative correlation between TNF-α and Complex-I subunits is significantly higher in basal breast cancer patients (Figure 12A) and no significant correlation in luminal breast cancer patients was observed. CIII subunits like UQCR10, UQCRB, and UQCRQ expression were also altered in basal breast

cancer patients which showed a significant negative correlation with TNF-α expression as compared to luminal breasts cancer patients (Figure 12B).

The survivability of breast cancer patients was checked using the Kaplan-Meier survival plot analysis. The high expression of subunits of mitochondrial complexes like NDUFB1 (p=0.052), SDHA (p=0.011), and COX7C (p=0.064) showed an increased percentage of survival and an increased number of survival days. (Figure 12C)

This evidence strongly suggests that TNF-α differentially regulates the mitochondrial subunits in luminal and basal breast cancer patients and determine the survival rate and span of the breast cancer patients.

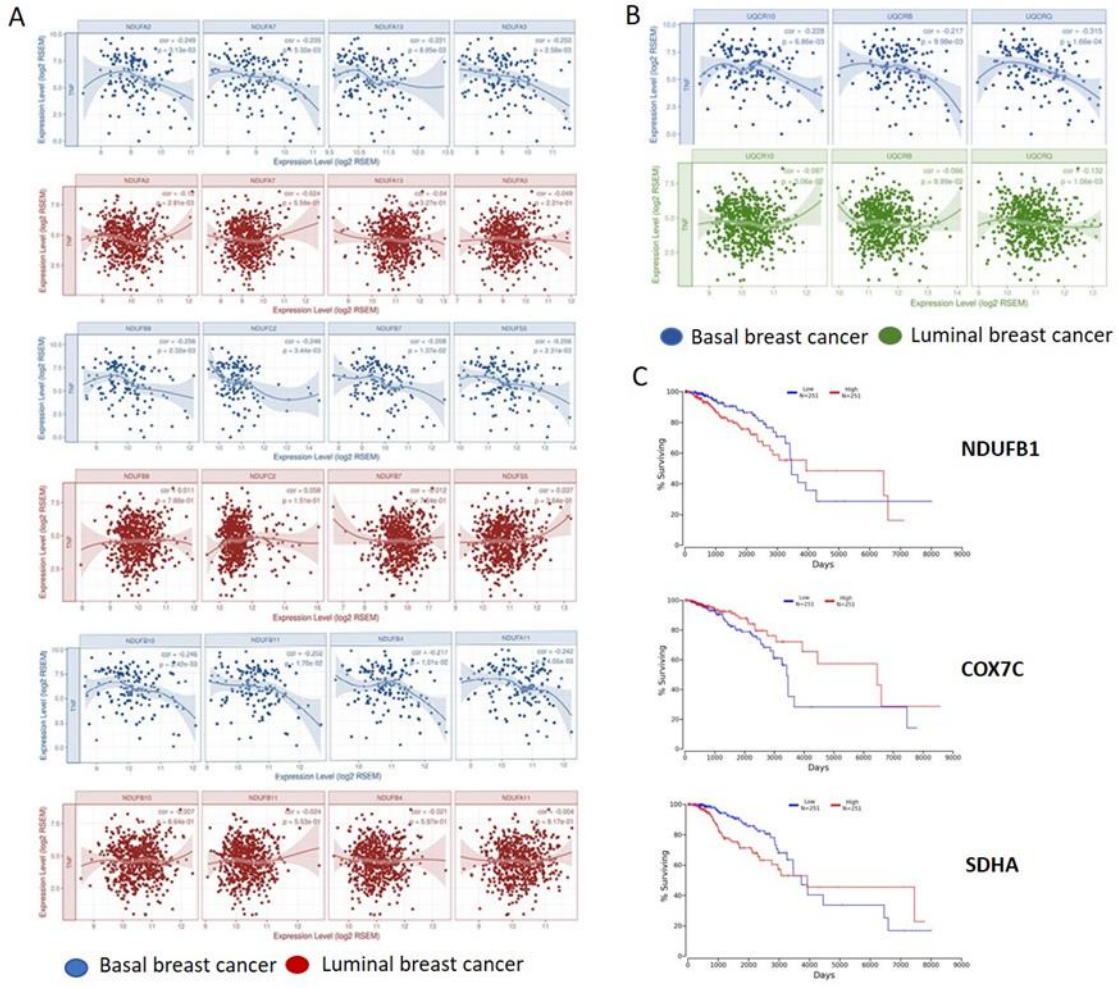


Figure 12: Subunit of complex negatively correlates with survival of the breast cancer patients (A)& (B) Gene expression analysis between TNF-α and mitochondrial CI and CIII proteins using TIMER database, respectively. Here blue indicates the gene expression in Basal and red/green indicates gene expression in Luminal

breast cancer patients. (**C**) Kaplan Meier survival plot of BRCA patients in low/high expression of mitochondrial NDUFB1, COX7C, and SDHA genes.

4.2.6 Discussion:

Cancer cells greatly depend on metabolic reprograming that contribute significantly to the changes that alter TME, metabolite availability, alterations in the immune cell metabolic pathways along with survival and growth [245, 246]. Differential metabolic adaption in the high level of TNF-α in TME of breast cancer cell types is not understood. Hence in the current section the study focussed on mitochondrial complex activities and metabolic adaptation and its role in determining the survival and clonogenicity in luminal A (MCF-7) and basal (MDA-MB-231) breast cancer subtypes.

The mitochondrial functional assays and metabolomics study revealed a differential status of mitochondrial functions and metabolic pathways that were regulated by TNF-α in both MCF-7 and MDA-MB-231 breast cancer cells. The decrease in complex-I activity in MDA-MB-231 cells showed altered mitochondrial OXPHOS which emphasized the need to understand its implications in breast cancer growth and metastasis. Studies have shown that mitochondrial complex I enhancement leads to decreased tumor growth and metastasis which is regulated by mTORC1 activity and autophagy in breast cancer cells [1, 247].

The study further focused on the assembly of mitochondrial respiratory chain complexes which are differentially regulated in MCF-7 and MDA-MB-231 cells. The level of mitochondrial DNA encoded transcripts specifically ND2 and ND3 which form part of the core unit of the complex-I, increased in MCF-7 whereas remaining unaltered in MDA-MB-231 cells. The levels of NDUFS3 (N module component) and NDUFB1 (the component of ND4 module) was significantly decreased in MCF-7 cells in the presence of TNF-α however, increased in MDA-MB-231 suggesting that TNF-α can differentially modulate the complex-I activity which is in consonance within gel activity and super complex assembly. Interestingly studies have shown that mitochondrial proteins like NDUFB9 downregulation are involved in promoting migration, invasion and proliferation of MDA-MB-231 cells [166].

The decrease in complex-I/III activity may increase the level of ROS in triple-negative aggressive breast cancer cells which may act as mitohormetic response rather than cell death. This observation is in consonance with a recent report where it had been observed that an increased level of ROS in selected aggressive breast cancer cells from TNBC patients can

induce mitohormetic response in modulating nuclear genes which help to survive in the hostile tumor microenvironment [248].

The complex-II of mitochondria also known as succinate dehydrogenase (SDH complex), is important for the cancer cell metabolism as it is part of TCA as well as respiratory chain. Succinate is shown to potent oncometabolite not only in breast cancer but other tumors conditions like paraganglioma, renal cell carcinoma, papillary thyroid cancers, adrenal neuroblastoma etc [4, 249]. The decreased SDH activity in ER/PR –ve (MDA-MB-231) leads to accumulation of succinate in presence of TNF-α as compared to MCF-7. This is also in consonance with earlier observation where decreased complex-II had been observed in the tumor cells as compared to normal human mammary epithelial cell [249]. The alteration of complex-II is known to provide dual advantage to the aggressive tumor (ER/PR-ve: MDA-MB-231 cells). Firstly, the accumulation of succinate, which is transported to cytosol, where it inhibits HIF-α prolyl hydroxylases in the cytosol. This leads to the stabilization and activation of HIF-1α in normoxic conditions leading to increased angiogenesis and tumor vasculature [250]. This is also favoured by the recent study where decrease in complex-III in Treg cells increased the level of metabolites succinate and 2-hydroxyglutarate (2-HG) and succinate which alters epigenetic regulation of DNA [251]. Recent studies have highlighted succinate as an important metabolite that can affect the coenzyme Q pool redox state in inner mitochondrial membrane and regulate electron supply, O2 levels and ATP status in the cell [252].

Along with succinate, accumulation of pyruvate and aspartate were observed in MDA-MB-231 cells in presence of TNF-α as compared to MCF-7. Studies have shown mitochondrial pyruvate, pyruvate carboxylase levels and PC-dependent anaplerosis to be increased in breast derived lung metastasis compared to primary breast cancers [253]. Lung environment played crucial role in activating PC-dependent anaplerosis in breast cancer cells that metastasis to lungs as compared to primary tumor.

Aspartate is an amino acid important in protein synthesis but majorly it is essential for nucleotide biosynthesis in proliferating cells [254]. Aspartate biosynthesis occurs in cells via glucose and glutamine, which fuels the TCA to generate intermediates like oxaloacetate. The aspartate biosynthesis in cells can also be driven through reductive glutamine metabolism in cells which have defects in mitochondrial dependent aspartate biosynthesis. The presence of TNF-α in tumor microenvironment is known to stabilize the HIF-α [255],which is known to enhance the generation of aspartate through reductive glutamine metabolism by regenerating

citrate to support growth and proliferation [256].The previous studies supports the increased levels of aspartate which may be due to increased reductive glutamine metabolism in MDA-MB-231 cells to enhance their growth and proliferation in presence of TNF-α.

Similarly, mitochondrial complex-III, which can accept electrons from both complex-I and complex-II through ubiquinone is critical for mitochondrial function. Recent studies have revealed dramatic differences in complex III between normal and aggressive metastatic breast cancer providing direct evidence of impaired mtOXPHOS complex III to breast tumorigenesis [257]. The study here showed a decrease in complex-III activity which correlates with mitochondrial proteomics data. The level of assembly factor LYRM7 decreased in MDA-MB-231 cells compared to MCF-7 cells. Emerging reports suggest that binding of HSC20 (co-chaperone) to the LYR motif of LYRM7 in a pre-assembled UQCRFS1-LYRM7 intermediate in the mitochondrial matrix facilitates Fe-S cluster transfer to UQCRFS1, hence assembly of complex-III(Chaperon CI-III). This decrease in LYRM7 in mitochondria of MDA-MB-231 cells strongly suggests that incorporation of Fe-S complexes in mitochondrial electron transport chain may be modulated in mitochondrial complex-III and super complex assembly. Hence TNF-α modulates flux of NADH which will shift the TCA cycle intermediates towards anaplerotic reactions in aggressive breast tumor cells (MDA-MB-231).

Further, the differential mitochondrial protein expression, mitochondrial functions along with varying metabolites levels led us to further explore their effects on survival and clone forming ability of MCF-7 and MDA-MB-231 cells. Studies have shown that TNBC cells have different metabolic features compared to ER positive cells [235]. Increased glucose uptake, lactate production and decreased mitochondrial respiration were observed as characteristic differences highlighting their dependency on glycolysis. Interestingly it was observed that TNF-α inhibited the clonogenic ability of MCF-7 cells which is rescued in presence of pyruvate suggesting that electron acceptors are limiting factor. Pyruvate level can be differentially regulated in MCF-7/MDA-MB-231 cells, which is known to act as an electron acceptor and can determine the cell proliferation. Interestingly, in MDA-MB-231, highly metastatic cells, pyruvate is not a limiting factor as TNF-α reprogrammed the activity of OXPHOS for anaplerotic reaction, the study observed that pyruvate supplementation showed no major effect on cell proliferation or clonogenic abilities of the cells. This observation further supported as high level of pyruvate was observed in MDA-MB-231 cells as compared to MCF-7 cells. In vivo studies using magnetic resonance spectroscopy have analysed the conversion of hyperpolarized 13C-pyruvate to 13C-lactate in two breast cancer cell lines of different malignancies (MCF-7,

MDA-MB-231) [258]. The results here show that both the cells have different conversion rates of pyruvate to lactate and are glucose/glutamine dependent.

The decrease in complex-I/III activity may increase the level of ROS in triple-negative aggressive breast cancer cells which may act as mitohormetic response rather than cell death. This observation is in consonance with a recent report where it had been observed that an increased level of ROS in selected aggressive breast cancer cells from TNBC patients can induce mitohormetic response in modulating nuclear genes which help to survive in the hostile tumor microenvironment.

The overall decrease in the mitochondrial proteins and complex activity in MDA-MB-231 is supported by a recent study where BTB and CNC homology1 (BACH1), a haem binding transcription factor increased in tumors from patients with TNBC which negatively regulates transcription of electron transport chain (ETC) genes. It was observed that enhancing the reliance of breast cancer cells to mitochondrial functions by modulating the transcription factor BACH1 using hemin which initiates degradation of BACH1, sensitizes the cancer cells to metformin. Recent studies have also identified inhibition of BACH1 in reducing migration and invasion of cancer cells by increasing mitochondrial metabolism [259].

Together with the differential mitochondrial proteome analysis, mitochondrial functions and metabolite levels obtained in the MCF-7 and MDA-MB-231 cells in presence of TNF-α allows to distinguish between Luminal A and TNBC tumors. In the current study, the TIMER webserver showed a negative correlation of CI and CIII subunits with TNF-α in basal breast cancer patients. This correlates with the decrease in complex assembly and activity in MDA-MB-231 in presence of TNF-α. Thus, the study highlights the role of TNF-α in differentially regulating the migration and clone forming ability of breast cancer subtypes. It also clearly shows that mitochondrial functions and metabolism plays a critical role in tumor growth and survival of breast cancer cells. The study draws attention to the metabolic alterations and metabolic reprogramming that are identified in both the cells which provide metastasis and invasion initiation advantages to the highly metastatic MDA-MB-231 cells as compared to MCF-7.

4.3 Study the role of TNF-α -regulated NF-κB in regulating LYRM7 in breast cancer

4.3.1 NF-κB subunits are differentially expressed in breast cancer patient and are prognostic to the survival outcome

NF-κB has been identified as a critical link between inflammation and cancer. NF-κB transcriptional factors are key regulators of cell survival, cell proliferation and cell death in many malignancies. Therefore, it is important to dissect the role of different subunits of NF-κB and explore their implications in breast cancer. To understand the role of NF-κB-mediated inflammation in the tumor microenvironment in various subtypes of breast cancer, various databases were explored. RELA, RELB, c-REL, NFKB1 and NFKB2 are the major NF-κB family of proteins, and their dimeric complexes determine the activation of this pathway under various physiological conditions. To investigate the potential role of the NF-κB family of proteins in breast cancer patients, their differential expression and correlation with survival was analysed. TIMER database was explored to analyse the various subunit of NF-κBand their differential expression in the tumor versus adjacent normal breast tissue (Figure 13A). A differential expression of RELA, RELB, REL, NFKB1 and NFKB2 was observed in tumors versus normal tissue in breast cancer patients. RELB and NFKB2 expression was significantly increased in tumor versus normal breast tissue, whereas a significant decrease was observed in *NFKB1* gene expression. The difference in RELA and cREL was not significant in tumor versus normal breast tissue but RELA was higher in Basal and Luminal A subtypes compared to HER2 and Luminal B. The analysis also showed that cREL was higher in Luminal A and Luminal B compared to Basal and HER2 subtypes of breast cancer patients.

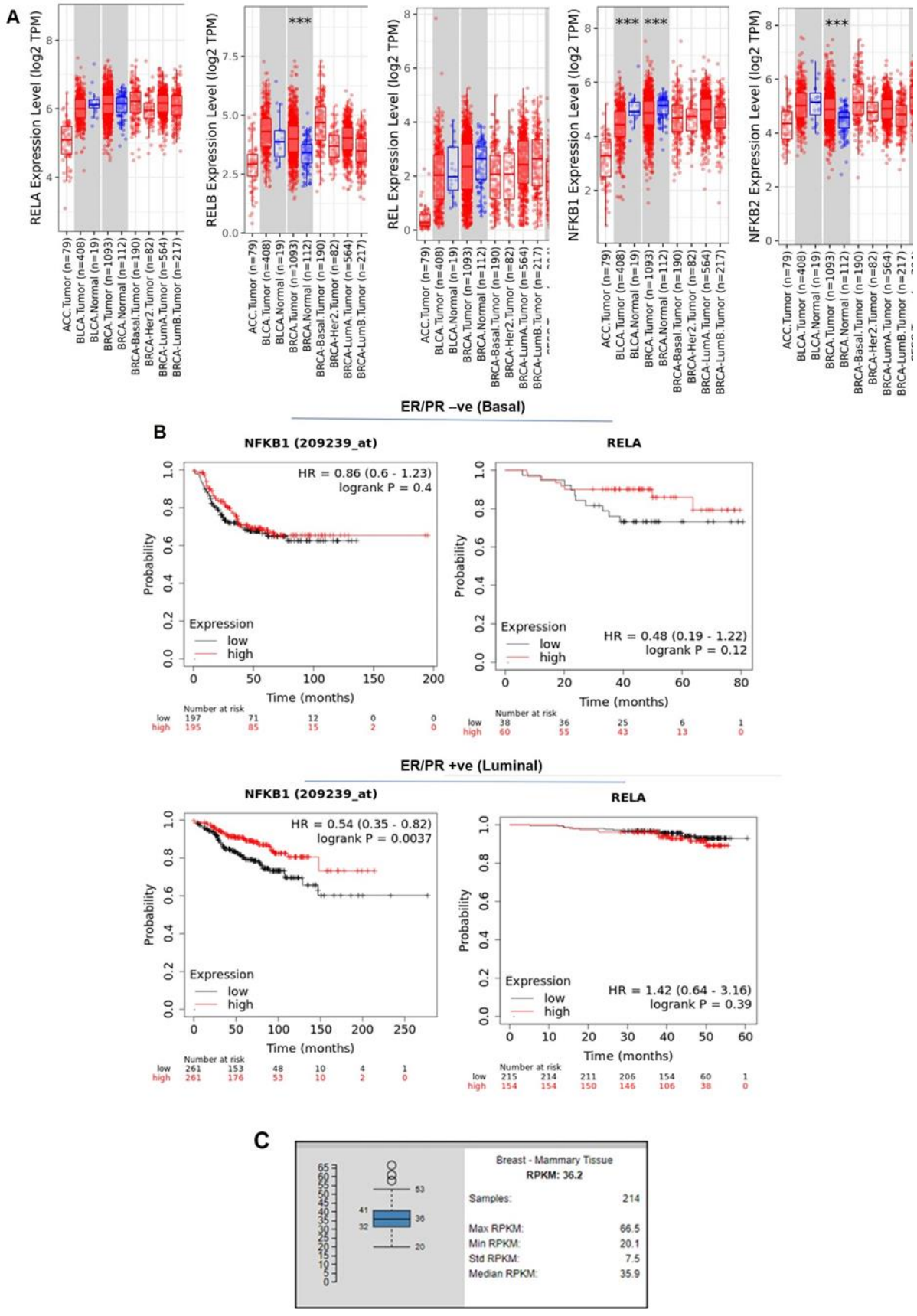


Figure 13: Expression of NF-κB subunits in breast cancer patients (**A**) RELA, RELB, cREL, NFKB1 and NFKB2 transcript levels in normal versus tumor tissue of breast cancer patients from the TIMER database. (**B**)

Survival probability of Basal and Luminal A subtypes of breast cancer patients with higher and lower expression of NFKB1 and RELA were analysed using the Kaplan–Meier method. (**C**) RELA gene expression in breast cancer patient samples compared to normal using TCGA database

Next, expression of these proteins in different subtypes of breast cancer patients using BCGENEX Miner database was also analysed (Figure 14). All the subunits showed differential expression in breast cancer patients with a different ER/PR status. A notable increase was observed in the expression of RELA, RELB and NFKB2 in ER/PR -ve (n=1551) as compared to ER/PR +ve (n=3242) breast cancer patients.

The formation of dimeric complexes of RELA with p50 protein (arise from NFKB1, a precursor protein) activates a major canonical pathway in cells. Thus, investigation of the clinical relevance of RELA and NFKB1 for survival in breast carcinomas with ER/PR +ve and -ve status was done using KM plot (Figure 13B). The KM analysis showed that higher expression of NFKB1 was associated with increased overall survival in Luminal A subtype (logrank P=0.0037, HR=0.54), but not in the basal subtype of breast cancer patients. The higher expression of RELA was associated with a distinct increase in overall survival in the basal subtype of breast cancer patients (logrank p= 0.12, HR=0.48), but not in Luminal A tumors.

Furthermore, the study investigated the expression of RELA, which is synthesized mainly as a mature protein, and which contains a TAD (Transactivation domain) that binds to the promoter region of NF-κB-responsive genes. Human Protein Atlas database was used to evaluate the expression of RELA in 214 mammary tissues and found that the expression was significantly high in tumor samples as compared to normal tissue (Figure 13C). A median of 35 RPKM (reads per kilobase of transcript per million reads mapped) was observed in breast cancer samples compared to standard 7.5 RPKM in normal samples. These results suggest that the differential expression of the NF-κB subunit could regulate NF-κB pathway in specific subtypes of breast cancer and could play an important role in breast cancer progression and survival of the patients.

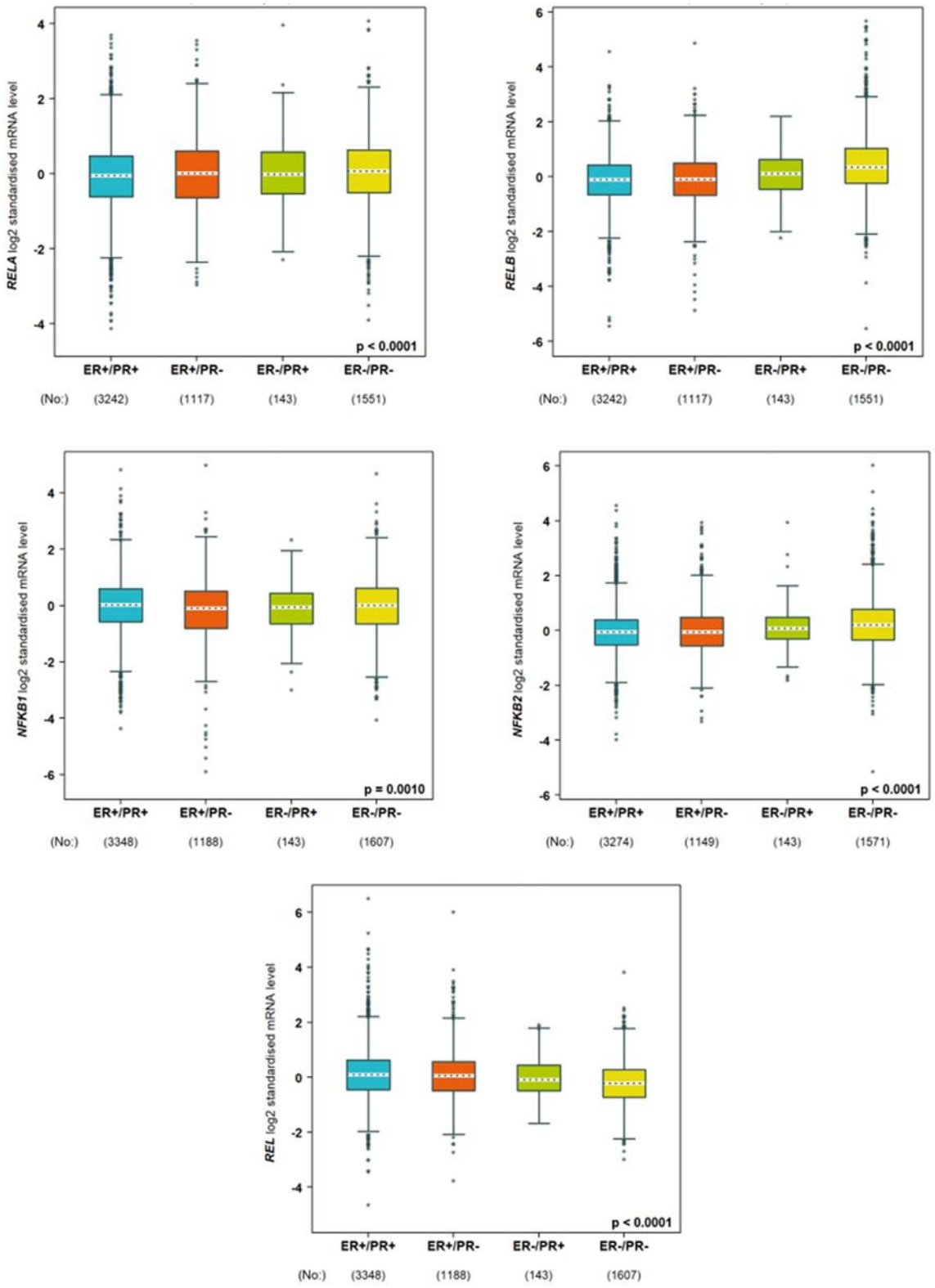
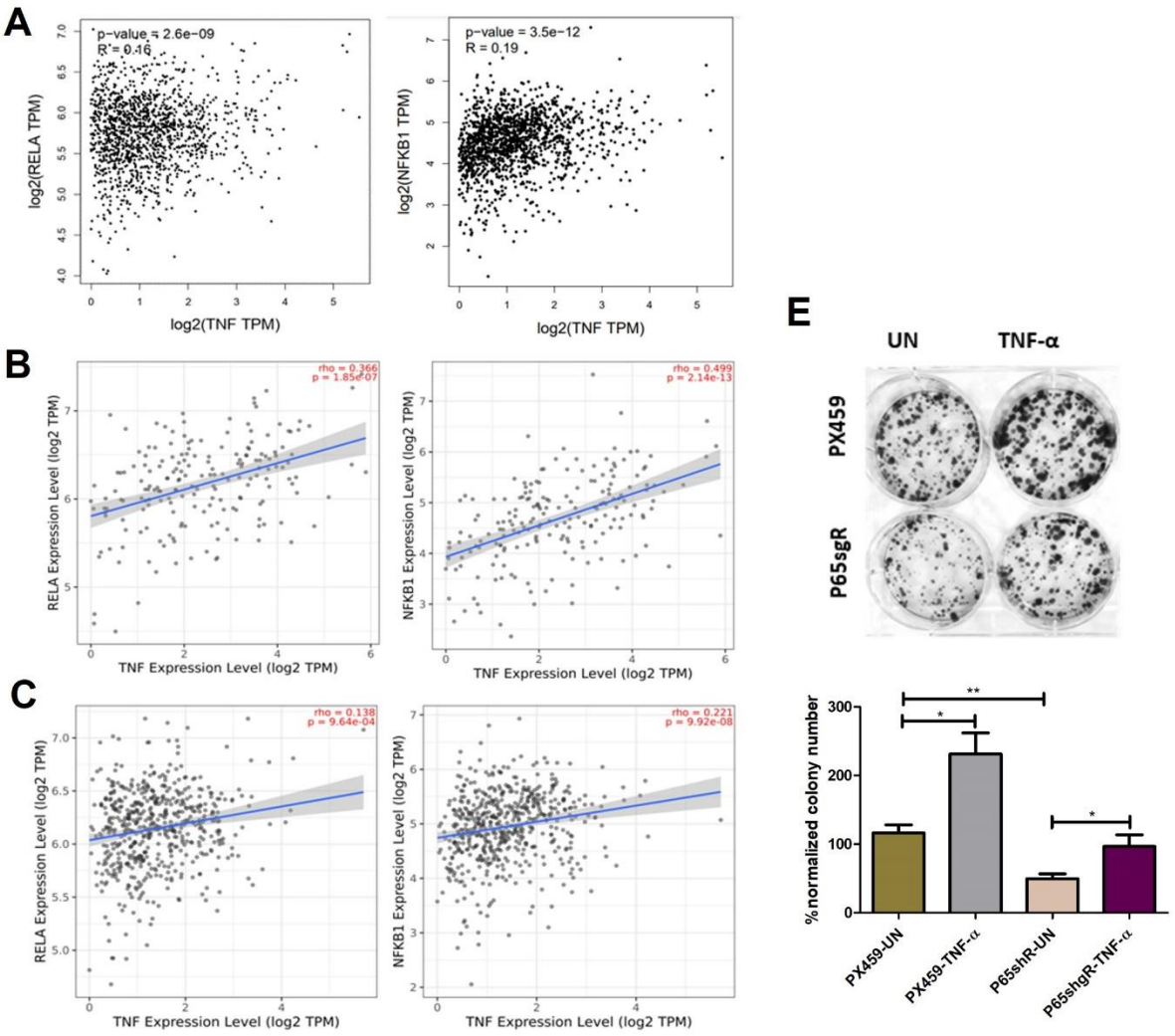


Figure 14: Differential expression of NF-κB subunits in subtypes of breast cancer patients using BCGENEX Miner Database

4.3.2 TNF-α and canonical pathway subunits are positively correlated in breast cancer patients and determine breast cancer cell survival:

The study analysed the correlation among TNF-α and RELA and NFKB1 genes and the association with breast cancer progression. Analysis of breast cancer patient samples showed a positive correlation between TNF-α with RELA and NFKB1 (R=0.16 and 0.19 respectively) (Figure 15A). A positive correlation was also observed in both basal as well as luminal breast cancer patients (Figure 15B&C). Further, the TIMER database was explored, and it was observed that the expression of RELA and NFKB1 was different in subtypes of breast cancer. A significantly higher positive corelation of TNF-α with RELA and NFKB1 was observed in the basal subtype of breast cancer as compared to other subtypes (Figure 15D). This suggest that canonical subunits positively correlate with TNF-α and could play crucial role in breast cancer tumorigenesis.

Further, the study analysed the effects of the NF-κB pathway on growth of MDA-MB-231 cells using colony forming assay. Transfection of cells with p65 (RELA) sgRNA and control vector was done and treated with TNF-α. TNF-α-treated MDA-MB-231 cells increased clonogenicity was compared to untreated vector transfected cells. p65 knockdown significantly inhibited clonogenicity of MDA-MB-231 cells compared to the vector control (Figure 15E). Further, TNF-α treatment to p65 sgRNA transfected cells showed an increase in the clone forming ability compared to untreated controls. These results show that TNF-α-induced NF-κB plays a critical role in tumorigenic potential of breast cancer cells.



D

cancer	RELA
ACC (n=79)	-0.032
BLCA (n=408)	0.279
BRCA (n=1100)	0.205
BRCA-Basal (n=191)	0.366
BRCA-Her2 (n=82)	0.1
BRCA-LumA (n=568)	0.138
BRCA-LumB (n=219)	0.172

cancer	NFKB1
ACC (n=79)	0.115
BLCA (n=408)	0.321
BRCA (n=1100)	0.216
BRCA-Basal (n=191)	0.499
BRCA-Her2 (n=82)	0.129
BRCA-LumA (n=568)	0.221
BRCA-LumB (n=219)	0.149

Figure 15: Correlation between TNF-α and canonical pathway subunits in breast cancer patients and breast cancer cell survival (**A**) Correlation between TNF-α and NFKB1 and RELA expression in breast cancer patients using the TCGA database (**B&C**) Correlation between TNF-α and NFKB1 and RELA expression in Basal and Luminal subtypes of breast cancer patients respectively using the TIMER database. (**D**) Correlation between TNF-α and NFKB1 and RELA expression in subtypes of breast cancer patients using the TIMER database. (**E**) MDA-MB-231 cells were transfected with vector control and p65 sgRNA and 24 h post transfection cells were seeded at 2000 cells/well. At 24 h post seeding, cells were treated with 10 ng/ml TNF-α and colony formation was measured at Day 12 of the treatment.

4.3.3 NF-κB regulates LYRM7 expression in breast cancer cells:

Reprograming of metabolism is important to meet the anaplerotic demands of highly proliferating and metastatic triple negative breast cancer cells. In previous proteomics study, it was shown that TNF-α induces differential expression of mitochondrial proteins in MCF-7 (less metastatic) and MDA-MB-231 (highly metastatic) breast cancer cells. This could be important for the differential proliferating capacity by modulating OXPHOS complexes activity and assembly. LYRM7, an assembly factor for complex III was increased in MCF-7 and MDA-MB-231 in presence of TNF-α. Since LYRM7 is important for the assembly of mitochondrial OXPHOS complexes, the role NF-κB was explored in the regulation of LYRM7 expression and its role in metabolic reprograming. Next the Eukaryotic Promoter Database was explored and found that the promoter region of LYRM7 has binding sites for NF-κB subunits. Promoter region of LYRM7 showed maximum binding site of LYRM7, subunit of NF-kB complex (Figure 16A&B). TIMER database also showed a positive correlation between RELA and LYRM7 in Basal and Luminal breast cancer patients (Figure 16C).

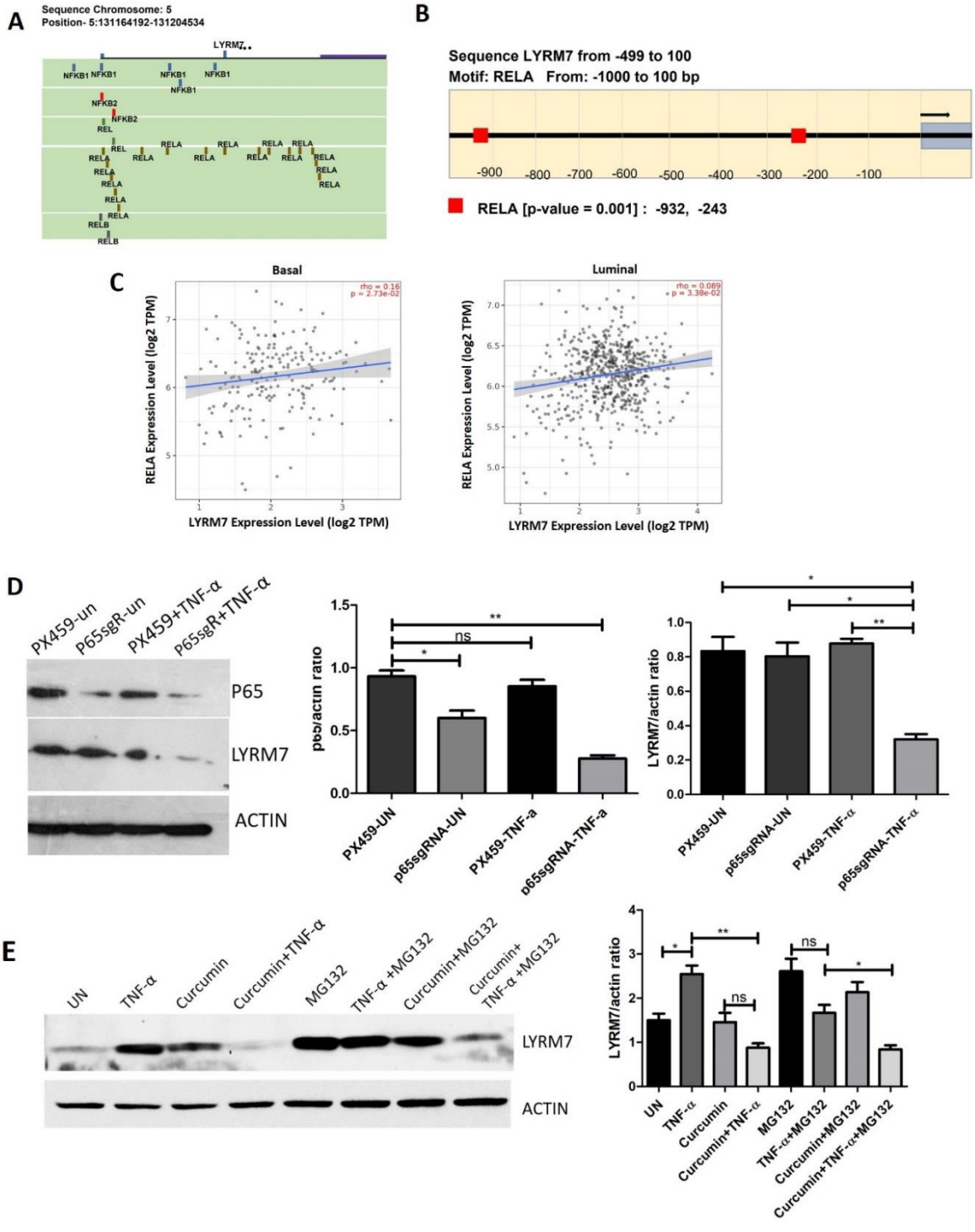


Figure 16: NF-κB increases LYRM7 expression in breast cancer cells(**A**&**B**) Analysis of binding sites for NF-κBsubunits and RELA on the promoter region of the LYRM7 gene using the Eukaryotic promoter database. (**C**) Correlation between RELA and LYRM7 gene expression in Basal and Luminal A subtypes of breast cancer patients using the TIMER database. (**D**&**E**) MDA-MB-231 cells were transfected with a vector control and p65 sgRNA and 24 h post transfection cells were treated with TNF-α (24h), MG132 (last 6 h) and Curcumin for 10h

as indicated. Protein levels for p65, LYRM7 and Actin were measured by western blotting using specific antibodies.

To establish further that NF-κB could control the expression of LYRM7 protein, a well-known chemical inhibitor of the NF-κB pathway was used and is known to decrease the phosphorylation of IκBα in cells. p65-GFP-transfected MCF-7 cells were treated with curcumin for 6h post transfection and checked for translocation of p65-GFP to the nucleus by fluorescent microscopy. Treatment with TNF-α for 10 h increased the translocation of p65-GFP to the nucleus in untreated cells. As a control, curcumin was used as an inhibitor and showed that this blocked p65 translocation to nucleus (Figure 17A). NF-κB activation leads to phosphorylation of IκBα in cells. The turnover of p-IκBα levels was analysed in presence and absence of curcumin and MG132 (Proteosome inhibitor). The levels of p-IκBα were reduced in presence of curcumin along with TNF-α compared to only TNF-α treated cells as expected (Figure 17B). These results confirm that the activation of NF-κB pathway is inhibited by curcumin. Further, p65sgRNA-transfected MDA-MB-231 cells showed decreased expression of LYRM7 in presence of TNF-α compared to untreated (Figure 16D). TNF-α-treated MDA-MB-231 cells showed increase in the expression of LYRM7, which significantly decreased in the presence of curcumin (Figure 16E). These results provide evidence that the TNF-α-induced NF-κB pathway regulates the LYRM7 expression and inhibition of this pathway leads to reduced expression of this gene in breast cancer cells.

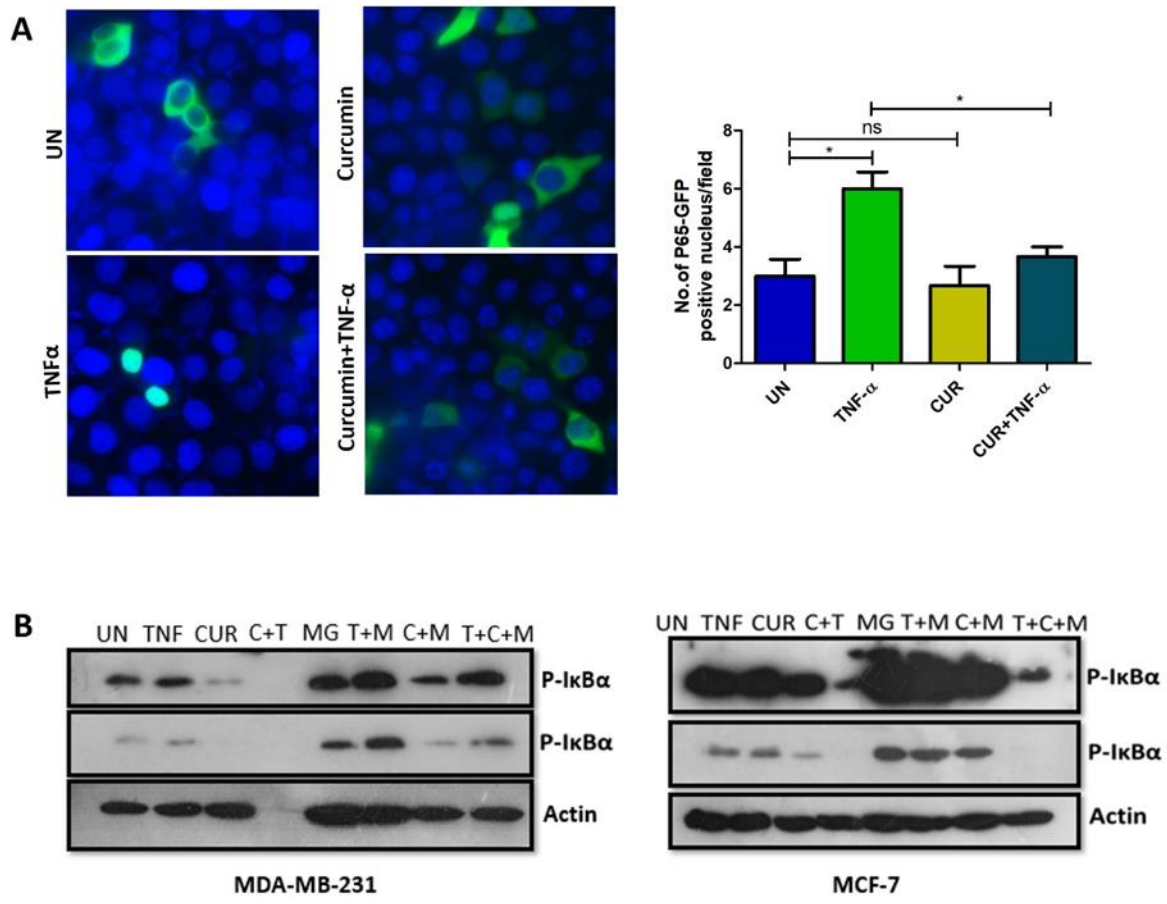


Figure 17: Inhibition of NF-κB by curcumin in breast cancer cell (A) p65-GFP transfected MDA-MB-231 cells were treated with TNF(10ng/ml) for 24hr and curcumin (10μM) for 6 hr and nuclear translocation was measured with florescent microscopy (B) MDA-MB-231 cells were treated with TNF (10ng/ml) for 24 h and curcumin (10μM) for 6 h and MG132 as indicated and pIκBα protein levels were measured using Actin as control by western blotting

4.3.4 LYRM7 expression regulates mitochondrial activities and complex assembly: Further to analyse the potential role of the *LYRM7 gene* in regulating specific mitochondrial functions in inflammatory conditions validated sgRNA was used to decrease its expression (Figure 18A & B).

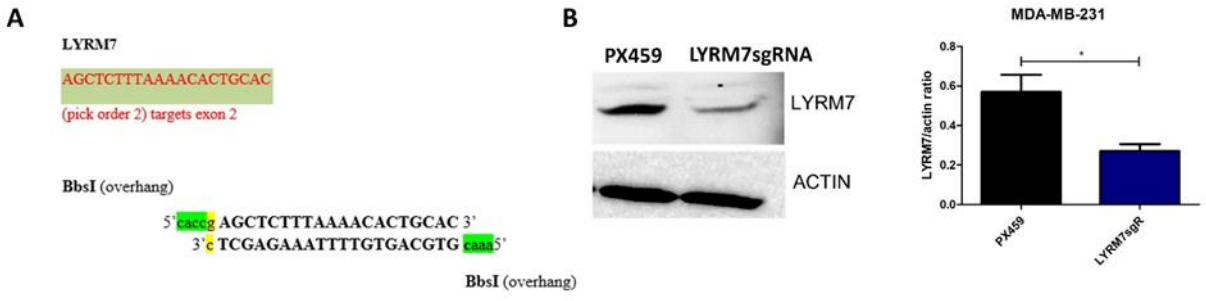


Figure 18: LYRM7 sgRNA validation in MDA-MB-231 cells (A) LYRM7sgRNA designing for CRISPR-Cas9 gene knockout. (B) Validation of LYRM7 knockout in MDA-MB-231 cells by western blotting.

Mitochondrial ROS was measured using MitoSOX inMDA-MB-231 cells. The level of mitochondrial ROS in MDA-MB-231 cells showed a significant increase in LYRM7 sgRNA transfection. MDA-MB-231 cells transfected with LYRM7 sgRNA showed decreased mitochondrial ROS levels in presence of TNF-α as compared to untreated. (Figure 19A). To further understand the effects of LYRM7 on overall cellular ROS generation, DCFDA staining was used to measure the relative intensity. There were no significant changes in cellular ROS levels in LYRM7 sgRNA transfected cells in presence and absence of TNF-α as compared to control cells (Figure 19B). Further, mitochondrial membrane potential also decreased in LYRM7 sgRNA transfected MDA-MB-231 cells as compared to the control. LYRM7 sgRNA transfected cells treated with TNF-α showed increased TMRM as compared to untreated cells (Figure 19C).

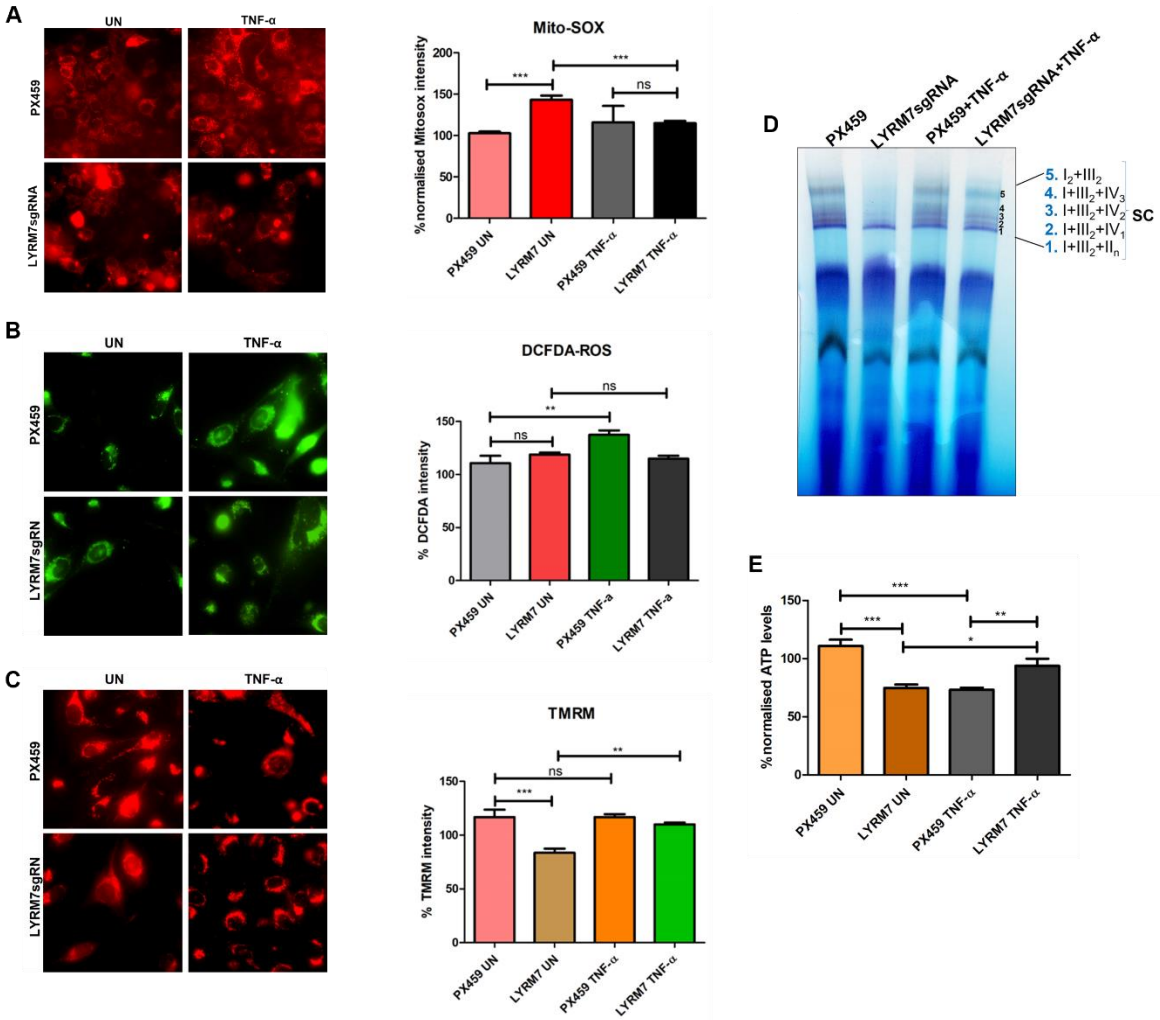


Figure 19: LYRM7 expression regulates mitochondrial activities and complex assembly under inflammatory condition MDA-MB-231 cells were transfected with vector control and LYRM7 sgRNA and 24h post transfection the cells were treated with TNF-α for 24 h. (**A**) MitoSOX staining for mitochondrial ROS was measured by fluorescence microscopy (**B**) DCFDA staining for cellular ROS was measured by fluorescence microscopy (**C**) TMRM staining for mitochondrial membrane potential was measured by analysing fluorescence intensity using fluorescence microscopy (**D**) Super-complex assembly and in-gel activity for CI and CIII was measured by Blue Native-PAGE (**E**) ATP levels were measured by a luciferase-based assay by measuring the luminescence in MDA-MB-231 cells

LYRM7 is a key protein, which acts as an assembly factor for Complex III. To characterize the effects of LYRM7 knockdown on mitochondrial super complex assembly and on complex activity, cells were transfected with the sgRNA and performed BN-PAGE for the analysis of the activity. Complex assembly was measured in digitonin solubilized mitochondria, and LYRM7 knockdown showed a severe defect in assembly of the respiratory super complex in both MCF-7 and MDA-MB-231 cells (Figure 19D). Surprisingly, knockdown of LYRM7 showed a rescue in the assembly of mitochondrial super complexes in the presence of TNF-α in MDA-MB-231 cells as compared to MCF-7 cells (Figure 20).

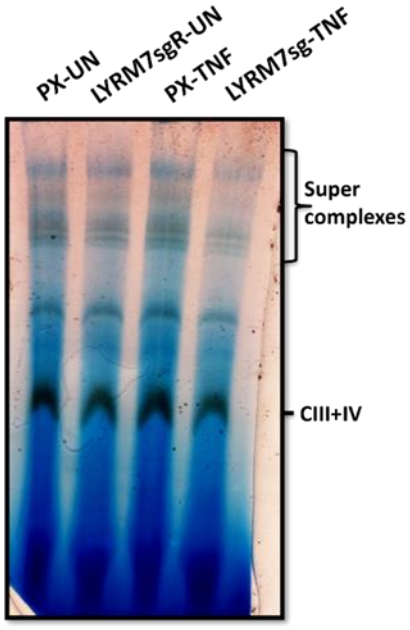


Figure 20: Supercomplex assembly and in-gel activity Complex I and complex III enzyme activity in LYRM7 sgRNA transfected MCF-7 cells in presence of TNF-α

The defect in complex assembly in the breast cancer cells due to decreased LYRM7 expression could affect ATP levels. The knockdown of LYRM7 in MDA-MB-231 cells showed a significant decrease in ATP levels (Figure 19E). These results collectively support the

conclusion that LYRM7 regulates the function of mitochondria in the presence of TNF-α in breast cancer cells.

4.3.5 LYRM7 expression regulates the migration, invasion and clone forming ability of metastatic breast cancer cells under inflammatory condition

Next investigation of LYRM7 regulated clonogenicity and migratory ability of MDA-MB-231 cells was done under inflammatory condition. Decreased expression of the *LYRM7 gene* in MDA-MB-231 cells increased the migration of these cells as compared to the vector control (Figure 21A). LYRM7 knockdown cells treated with TNF-α showed a significant decrease in migration as compared to untreated MDA-MB-231 cells. Clonogenicity of the MDA-MB-231 cells in LYRM7 knockdown conditions was further analysed. The clonogenic ability of MDA-MB-231 cells enhanced significantly after LYRM7 knockdown as compared to the vector control. MDA-MB-231 cells transfected with LYRM7 sgRNA and treated with TNF-α, showed no significant changes as compared to the untreated cells (Figure 21B). Next, invasion potential of LYRM7 sgRNA-transfected cells was measured and an augmented invasion compared to the vector control was observed. LYRM7 sgRNA-transfected cells showed decreased invasion in presence of TNF-α as compared to vector control cells (Figure 21C).

Collectively these results support the conclusion that *LYRM7* is an essential gene to maintain the homeostasis of healthy mitochondria and regulate the ROS levels, which can add to the survival and migration potentials of breast cancer cells under inflammatory conditions. In addition, TNF-α-mediated NF-κB activation can regulate the expression of key nuclear encoded mitochondrial proteins, which can alter ROS level and metabolic adaptation, hence ultimately effecting the cell growth and proliferation.

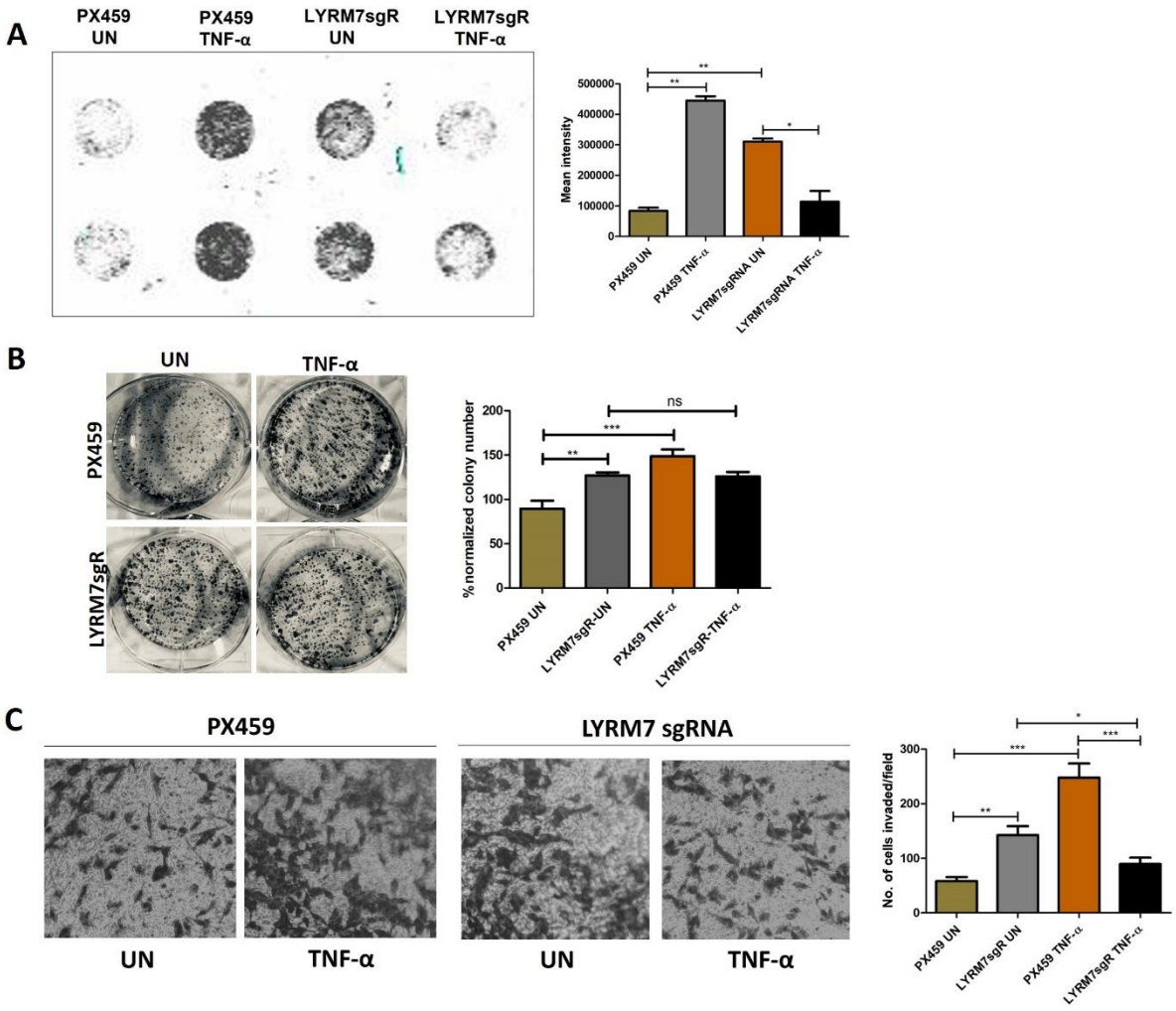


Figure 21: LYRM7 expression increases the migration, invasion and clone forming ability of metastatic breast cancer cellsMDA-MB-231 cells were transfected with vector control and LYRM7 sgRNA and 24h post transfection cells were treated with TNF-α as indicated (**A**). Transwell assay for migration of MDA-MB-231cells transfected with PX459 and LYRM7sgRNA in presence and absence of TNF-α (**B**). 2000 cells were seeded in 6 well plates and colony number was measured at Day 12 post seeding and treatment (**C**) Cell invasion ability was measured by staining the invaded cells at the bottom of the filter by crystal violet and imaging by microscopy at 10x magnification

4.3.6 LYRM7 expression decreases in Infliximab-treated mice:

To explore the effect of TNF-α on LYRM7 expression in vivo, syngeneic orthotopic mouse model of breast cancer was used. This involved the injection of 4T1 mouse breast cancer cells into a breast fat pad of BALB/c mice (Figure 22A). Mice were then divided in two groups and were treated with Infliximab, a chimeric monoclonal antibody against TNF-α. A decrease in the NF-κB responsive genes like TNF-α, IL-6 and IL-8 in Infliximab group of mice was

observed compared to the controls (54). The phosphorylated IKBα levels also decreased in Infliximab group of mice compared to control by western blotting. These results demonstrate a decreased activation of the NF-κB pathway in Infliximab-treated mice. Further, IHC analysis of these samples showed that LYRM7 expression was significantly decreased in Infliximab group compared to the controls (Figure 22B). A decrease in mRNA levels of LYRM7 was also observed by RT-PCR in the Infliximab-treated mice compared to controls (Figure 22C). Furthermore, there was a decrease in protein expression levels of LYRM7 primary tumor in the Infliximab group compared to controls (Figure 22D). Overall, these results indicate that TNF-α-induced NF-κB signaling is essential to express LYRM7 in the cells under inflammatory conditions in breast tumors.

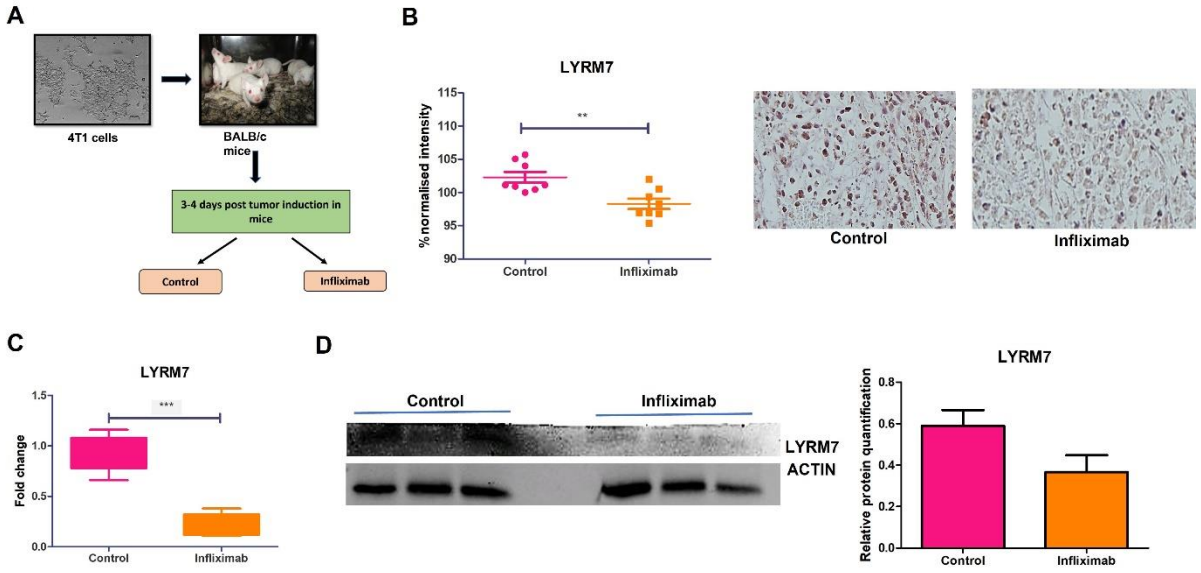


Figure 22: LYRM7 expression decreases in Infliximab injected mice: (**A**) Schematic representation of animal experiment in BALB/c mice using 4T1 breast cancer cells. (**B**) Sections for Tumor tissue in Control and Infliximab were immunohistochemically stained for LYRM7 and imaged by microscopy. (**C**) LYRM7 gene expression in control versus the Infliximab group of mice using qRT-PCR. (**D**) Protein levels of LYRM7 of control versus Infliximab group of mice using specific antibodies by western blotting.

4.3.7 LYRM7 expression decreases in metastatic breast cancer patients:

To further explore the clinical significance of LYRM7 in breast cancer patients LYRM7 mRNA levels were examined in tumors versus non-tumor mammary tissues using the TIMER database. LYRM7 expression was significantly lower in tumors compared to normal tissue (Figure 23A). Next, it was observed that the expression of LYRM7 was different in subtypes of breast cancer (Figure 23B). LYRM7 expression was significantly decreased in ER/PR negative (n=2728) as compared to ER/PR positive (n=1176) breast cancer patients (Figure

23C). Kaplan-Meier survival analysis indicated that high expression of LYRM7 in ER/PR positive and negative breast cancer patients showed poorer overall survival (Figure 23D). Next, analysis of the LYRM7 gene expression in breast cancer patients using TNM plot database showed a decreased expression in the metastatic and tumor compared to normal tissue (Figure 23E). These results indicate that LYRM7 expression could have clinical relevance in breast cancer. Further, the study also determined the mRNA and protein levels of LYRM7 in different subtypes of breast cancer patient samples. A decrease was shown in the TNBC subtype compared to others (Figure 23F&G). This suggests that the LYRM7 gene expression varies in subtypes of breast cancer and may hold clinical significance in determining the metastatic potential of breast cancer cells as well as survival outcome of the patient.

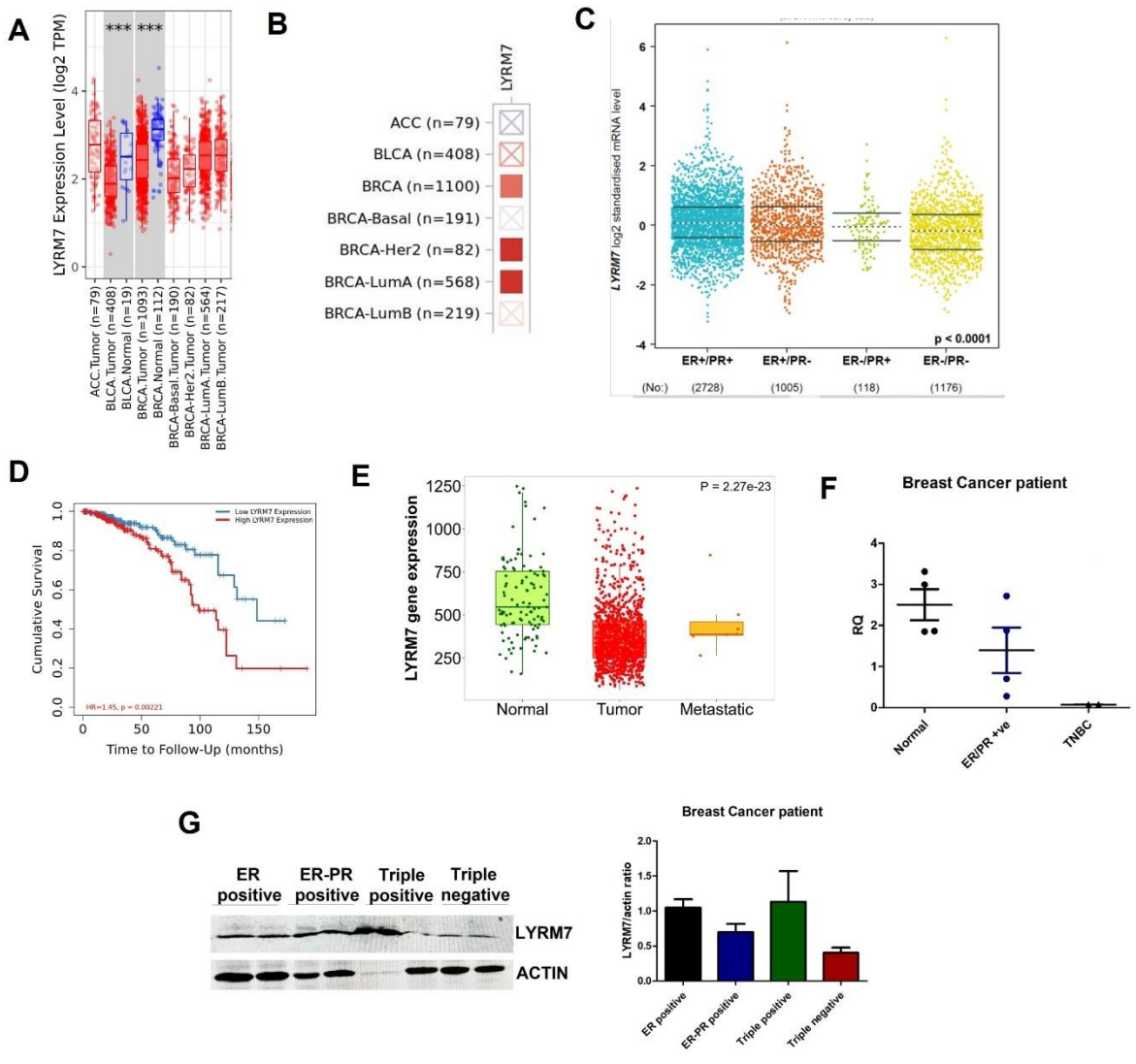


Figure 23: Expression of LYRM7 in breast cancer patients and their survival ability: (**A**) LYRM7 transcript levels in normal versus tumor tissue of breast cancer patients from the TIMER database. (**B**) LYRM7 gene expression in subtypes of breast cancer patients using the TIMER database. (**C**) LYRM7 gene expression in breast

cancer patients with varying ER/PR status using data from the BCGENEX Miner database. (**D**) Survival probability of Basal breast cancer patients with higher and lower expression of LYRM7 were analysed using the Kaplan–Meier method. (**E**) LYRM7 gene expression in metastatic patients versus normal using TNM plot database. (**F&G**) LYRM7 gene expression in breast cancer patients by qRT-PCR and western blotting using specific primers and antibody against LYRM7, respectively.

4.3.8 Discussion:

Chronic inflammation contributes to tumor initiation and progression in breast and many other cancers [260, 261]. The TME of solid tumors show immune cells infiltration and distinct pattern of pro-inflammatory cytokines including increased TNF-α, which activate various inflammatory pathway involving NF-κB [262]. TNF-α is a major cytokine that regulates the activation of this pathway under various physiological conditions [185, 263, 264]. Previous reports from our group have explored the importance of TNF-α in cell death and metabolism of breast cancer cells; however, TNF-α induced NF-κB activation and its role in the regulation of mitochondrial function and its implication in breast cancer progression remains elusive and needed further investigation [91, 265, 266]. Here a systemic investigation of the expression of different NF-κB subunits in various subtypes of breast cancer patients was done. The study demonstrated that the levels of subunits were different in subtypes of breast cancer and their expressions also regulates LYRM7, reprograming metabolism. This is prognostic for the survival of breast cancer patients.

RELA is predominantly expressed in different subtypes of breast cancer, which suggests an important role in breast cancer progression [267, 268]. TNBC patients, who have undergone adjuvant chemotherapy, showed RELA and NFKB1 overexpression and doxorubicin resistance [269]. Recent study has identified the anti-tumor effects of inhibiting RELA nuclear translocation in TNBC cell lines MDA-MB-231 and MDA-MB-468 along with and a human TNBC xenograft mode[90]. CRL1101, an allosteric inhibitor binds directly to RELA and blocks its nuclear translocation and significantly reduced breast tumor growth. RELA, which is major subunit of NF-κB is responsible for recruiting CECR2 to target genes which promote metastasis in breast cancer [270]. Metastatic breast cancer cell lines like MDA-MB-231 cells have an open chromatin configuration as compared to less metastatic cell lines. This helps in the direct binding of the NF-κB/RELA complex to increase transcription of proto-oncogenes like Ets1 [270]. Piao et al. identified α-catenin acts as tumor suppressor that inhibits NF-κB signaling and sequesters RELA/p50 in the cytoplasm leading to decreased expression of TNF-

α, IL-8 and RELB in E-cadherin-negative basal-like breast cancer [271]. The study targeted RELA, the major subunit, which forms a dimeric complex with p50 and activates NF-κB to induce genes related to cell survival, cell death and inflammation. A positive correlation was observed between TNF-α and the RELA subunit in breast cancer patients, which highlights the influence of NF-κB in regulating the survival of breast cancer cells and its implications in breast cancer migration and progression. Previously, the work demonstrated the effect of TNF-α on regulating mitochondrial protein expression in ER/PR positive (MCF-7) and ER/PR negative (MDA-MB-231) breast cancer cells. TNF-α functions in a dual manner regulating either cancer cell survival or death depending on the levels in the tumor microenvironment. This fine tuning between its expression and effects in TME can support the growth and metastasis of cancer cells to distant organs. In the current study, the influence of TNF-α-induced NF-κB on the expression of LYRM7 was shown, which was expressed less in triple negative breast cancer cells compared to luminal in presence of TNF-α.

The consequences of changes in the expression of mitochondrial proteins in responses to inflammation on metabolic reprograming in cancer including breast cancer is not well understood. Analysis of mitochondrial proteome showed increased expression of LYRM7, in presence of TNF-α in ER/PR positive compared to negative breast cancer cells. However, its significance in breast cancer progression and survival outcome has not been explored. The study demonstrated that *LYRM7* is an NF-κB-regulated gene, which shows a positive correlation with *RELA gene* expression in breast cancer patients. Further, it was observed that LYRM7 affected the assembly of super complexes in MDA-MB-231 cells as compared MCF-7. The activity of complex III was also decreased in an LYRM7 knockdown condition shown by the in-gel staining. Further observed a rescue was observed in the super complex assembly in presence of TNF-α in MDA-MB-231 cells. The effect can be rescued because TNF-α induces NF-κB and can rescue LYRM7 expression. LYRM7 is important factor, which is essential for the assembly of mitochondrial OXPHOS super complex [272]. LYRM7 is essential for transfer of Fe-S clusters to complex-III in association with UQCRFS1, which is a crucial step in assembly of complex-III. Knockdown of LYRM7 disrupted complex III activity and disturbed the higher order CI-CIII formation, which is in consonance with our current study. LYRM7 has been identified as a novel disease-related gene responsible for causing CIII defects and severe mitochondrial encephalopathy. Mutations in the LYRM7 gene cause serious pathogenicity and this confirms its role in the terminal step of CIII assembly. Exome sequencing studies identified a 4 base pair deletion in LYRM7, which causes reduced amounts of Rieske Fe-S protein, and

this was responsible for leukoencephalopathy. Targeted sequencing and brain magnetic resonance imaging in selected patients having leukoencephalopathy and complex III deficiency showed homozygous mutations in LYRM7. In the current study, the role of LYRM7 on mitochondrial functions was explored in breast cancer cells. A decrease in ATP production in cells with defects in mitochondrial complex assembly was observed. The decrease also showed enhanced ROS production and decrease in the mitochondrial membrane potential in MDA-MB-231 breast cancer cells with an LYRM7 knockdown. Interestingly, TNF-α rescued mitochondrial ROS generation in sgRNA transfected both MDA-MB-231 and MCF-7 cells. These results suggest that TNF-α-induced NF-κB activation can enhance the expression of LYRM7 and hence rescue mitochondrial functions. Previous reports from our groups and others clearly show that NF-κB regulated mitochondrial functions are responsible for driving cell survival in various disease models [185, 273].The present study demonstrated that inhibiting TNF-α actions by Infliximab, a chimeric monoclonal antibody, decreased the expression of the LYRM7 gene in a 4T1-BALB/c mouse model of breast cancer. A decrease in the RELA expression and mitochondrial LYRM7 protein expression was also validated in the Infliximab-treated group of mice with breast tumors compared to control mice and this was associated with a 60% decrease in metastasis to the lungs. The current study also revealed that expression of the assembly factor, LYRM7, is decreased in breast tumors compared to normal tissue. Further it was analysed that the expression is significantly decreased in ER/PR negative patients as compared to ER/PR positive. Next, the study analysed the gene expression of LYRM7 in breast cancer patients and observed a significant decrease in the ER/PR -ve group compared to ER/PR +ve. The current study established that TNF-α-induced NF-κB expression plays an essential role in the TME of solid tumors and could determine tumor progression in breast cancer patients. Interestingly, TNF-α activation of NF-κB regulates LYRM7 expression in a stoichiometry ratio to regulate complex activity for metabolic adaptation for the highly proliferating malignant cells. Studies related to defects in mitochondrial complexes have shown the potential as a target for therapeutics. Mitochondrial complex-I has been identified as major complex involved in regulation of NAD+/NADH ratios and this can control breast cancer progression and metastasis. Antonio et al. showed that enhanced expression of yeast NADH dehydrogenase Ndi1 in cancer cells restored the redox balance and complex-I defects and inhibited tumorigenesis. In a recent study, it was observed that complex-I mediated reprogramming of critical metabolite like formaldehyde can rescue the cancer cells from

genotoxic stress. Hence reprogramming of metabolism could also provide chemotherapy resistance in patients however this needs be explored further in breast cancer.

Therefore, it is important to monitor the complex assembly factors, which can determine critical metabolic adaptations during tumor progression. TNF-α-induced NF-κB activation and effects on LYRM7 should be investigated in breast cancer and other cancer patients. It will also be important to understand LYRM7 modulation during the chemotherapy and post-surgery for developing targeted therapy. This will also help to develop a strategy to rewire metabolism from anabolic pathways towards OXPHOS activity for inhibiting metastasis and chemotherapy resistance and relapse in breast cancer.

4.4 Study the role of TNF-α -regulated NF-κB in regulating Autotaxin (ATX) in breast cancer

4.4.1 Infliximab mediated inhibition of TNF-α signaling inhibit breast cancer metastasis to lungs

TNF-α is a major inflammatory cytokine in the tumor microenvironment and its levels are increased in metastatic breast cancers and adversely affects disease progression. The activation and in activation of multiple signaling pathways drive the tumorigenic potentials of breast cancer cells with the TME. TNF-α mediated regulation of autotaxin-LPA is emerging contribute to tumor progression however it had not been systemically investigated. Autotaxin-LPA signaling is exploited by the cancer cells to maintain tumor growth, enhanced angiogenesis and migration and invasion of cancer cells. So, the study part focussed focused on targeting TNF-α signaling in a syngeneic BALB/c mouse model using 4T1 breast cancer cells using Infliximab, a monoclonal antibody against TNF-α and its effects on ATX activity in breast cancer. Infliximab, was injected in BALB/c mice on day 7, 13, 21 after the inoculation of 4T1 breast cancer cells into a mammary fat pad (Figure 24A). Tumor volumes (Figure 24B) appeared to be decreased marginally in the Infliximab-treated mice compared to the controls. The mass of the excised tumors, which is a more accurate measurement, was significantly decreased in the Infliximab group of mice compared to the controls (Figure 24C). Our major finding was a remarkable decrease of ~60% in the number of lung metastatic nodules in the Infliximab group of mice at day 24 of the experiment (Figure 24D-E). A significant decrease was observed in metastatic and proliferation markers in the tumors of Infliximab group of mice compared to Control mice by IHC using antibodies against Ki-67 (cell proliferation) and Vimentin (epithelial-mesenchymal transition (EMT) (Figure 24F-G). Thus, Infliximab can decrease the breast tumor growth, but the more important finding was the decrease in lung metastasis.

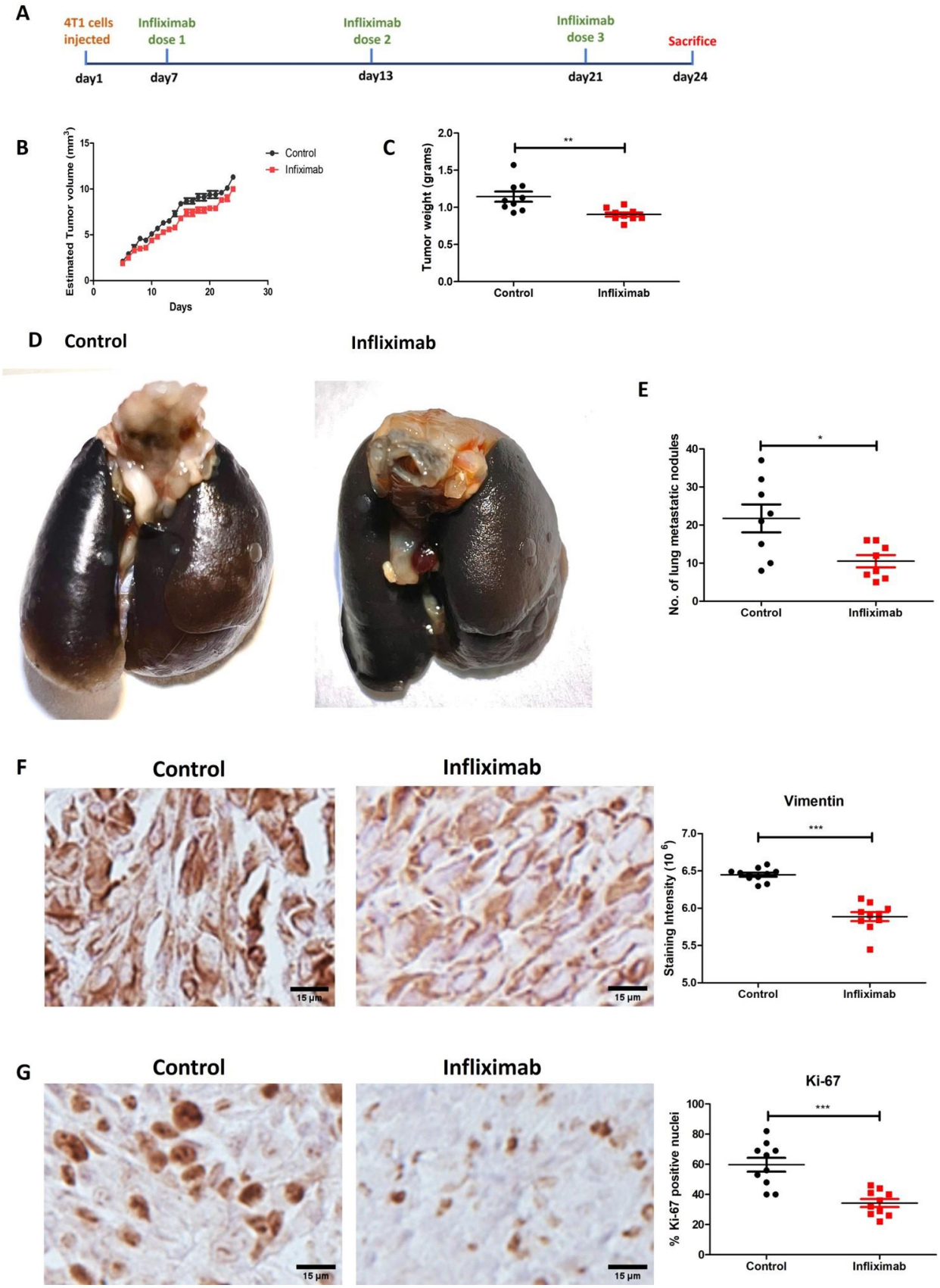


Figure 24: Infliximab decreases breast cancer metastasis to lungs(A) Schematic representation of study protocol, **(B)** Tumor volume measured using callipers in control and Infliximab groups of mice,**(C)**Tumor mass

excised from the Control and Infliximab groups of mice on day 24 **(D)** Metastatic lung nodules in lung injected with India Ink, **(E)** Number of lung metastatic nodules in control versusthe Infliximab treated miceand (**F&G**) Expression of Vimentin and Ki-67 by IHC staining in Control and Infliximab treated tumor..

4.4.2 Infliximab alters the concentrations of cytokines in the plasma and breast tumors of syngeneic mice model

TNF-α-induced NF-κB pathway is a one of the major inducers of inflammatory cytokines in breast cancer. Therefore, the concentration of cytokines/chemokines/growth factors in plasma and the primary tumor of Infliximab-treated mice compared to control mice were evaluated. Infliximab treatment decreased the levels of TNF-α, IL-4 and MCP-1 in plasma compared to the controls (Figure 25A). A significant increase was observed in the plasma levels of IFNγ in the Infliximab group of mice compared to the controls. Primary tumors showed a significant decrease in IL-4 and IL-12p40 in Infliximab-treated mice (Figure 25B).

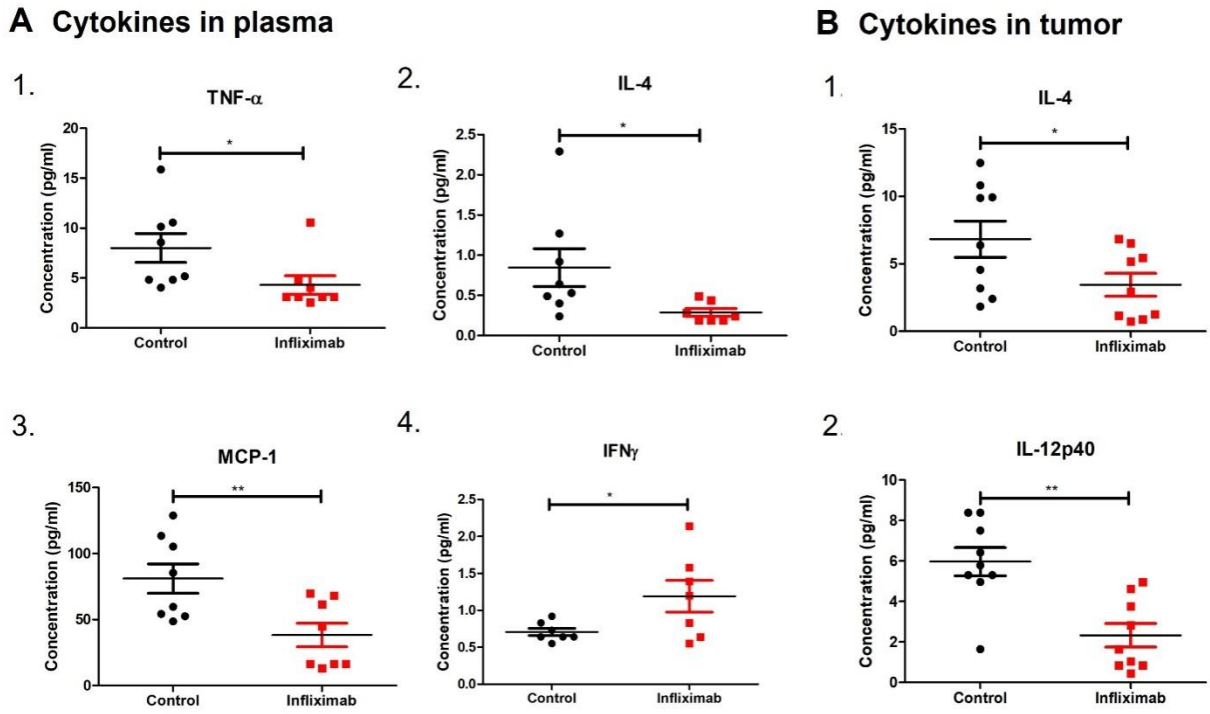


Figure 25: Cytokine profile in BALB/c mice treated with Infliximab (A) Concentration of TNF-α, IL-4, MCP-1 and IFNγ in plasma of BALB/c mice treated with Infliximab and control **(B)** Concentration of IL-4 and IL-12p40 in primary tumor of BALB/c mice treated with the Infliximab and control groups.

4.4.3 Infliximab treatment decreases NF-κB activation in breast tumors of syngeneic mice model

TNF-α binds to its receptor TNF-R1 and activates NF-κB pathway which regulates several key pathways essential for tumor progression. The effect of Infliximab was explored on NF-κB and observed a decreased activation in tumors of Infliximab group of mice compared to the control group. NF-κB-responsive genes such as TNF-α, IL-6 and IL-18 showed significant decreases in the mRNA levels in the primary tumors of Infliximab-treated mice compared to the controls. A significant decrease was also observed in the RELA mRNA levels, the main subunit of NF-κB dimer p65/p50 responsible for activation of the pathway (Figure 26A). A significant decrease in the mean intensity was observed by immunohistology staining using antibody against RELA (p65). Infliximab-treated mice also showed a decreased translocation of p65 from cytoplasm to the nucleus (Figure 26B). Phosphorylation of IκBα by IKK complex initiates its degradation and activates nuclear translocation of p65, which is a major event in the activation of NF-κB. The level of 36 kDa band of the phosphorylated form of IκBα decreased, which indicates decreased proteasomal degradation and decreased activation of NF-κB (Figure 26C). Altogether, these results demonstrate that Infliximab treatment inhibits NF-κB activation compared to the controls.

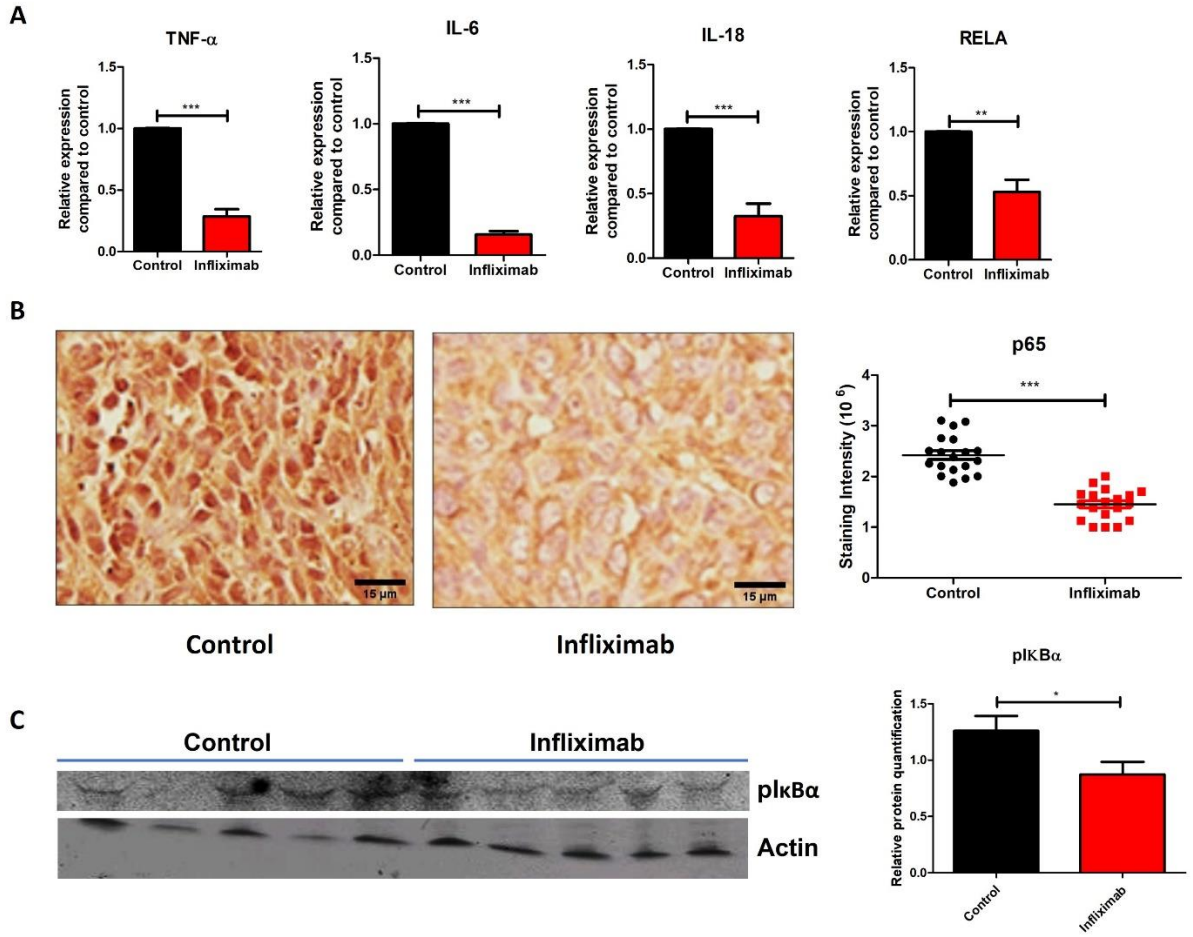


Figure 26: Infliximab inhibits NF-κB activation in breast tumors (A) mRNA expression for TNF-α, IL-6, IL-18 and RELA in Infliximab treated and control mice. (**B**) Representative images of IHC staining using specific antibody for RELA and nuclear staining by hematoxylin in Infliximab treated mice compared to controls, (**C**) Immunoblot showing levels of pIκBα and actin using specific antibodies by western blotting in the tumor sections of Infliximab group of mice compared to controls.

4.4.4 TNF-α, RELA and ATX are positively correlated in breast cancer patients

Inflammation in cancer is driven by various cytokines, chemokines, and growth factors, which regulate signaling in the TME to meet the growing requirements for cancer cells. The study further investigated whether the TNF-α-regulated NF-κB pathway can control the expression of ATX, which is one of the upregulated genes in breast cancer. Further, various databases were explored to correlate the expression pattern of these candidate genes in breast cancer. Timer database which is used for analysing the correlation between genes in tumor, showed a positive correlation between TNF-α and ENPP2 expression in different subtypes of breast cancer (Figure 27A & B). Moreover, there was a positive correlation between RELA (major NF-κB

subunit) and ENPP2 using The Cancer Genome Atlas (TCGA) database (Figure 27C). The BCGenex Minor Database was analysed for expression of ENPP2 in breast cancer patients with varying ER/PR status (Figure 27D). ENPP2 expression in tumors was significantly higher in ER/PR -ve subtype (n=1551) in comparison to ER/PR +ve subtype (n=3242). This suggested that the expression of ATX increased in a highly metastatic subtype of breast cancer. Thus, these results showed that there were positive correlations among TNF-α, RELA and ATX that can be investigated further to understand their roles in breast cancer metastasis.

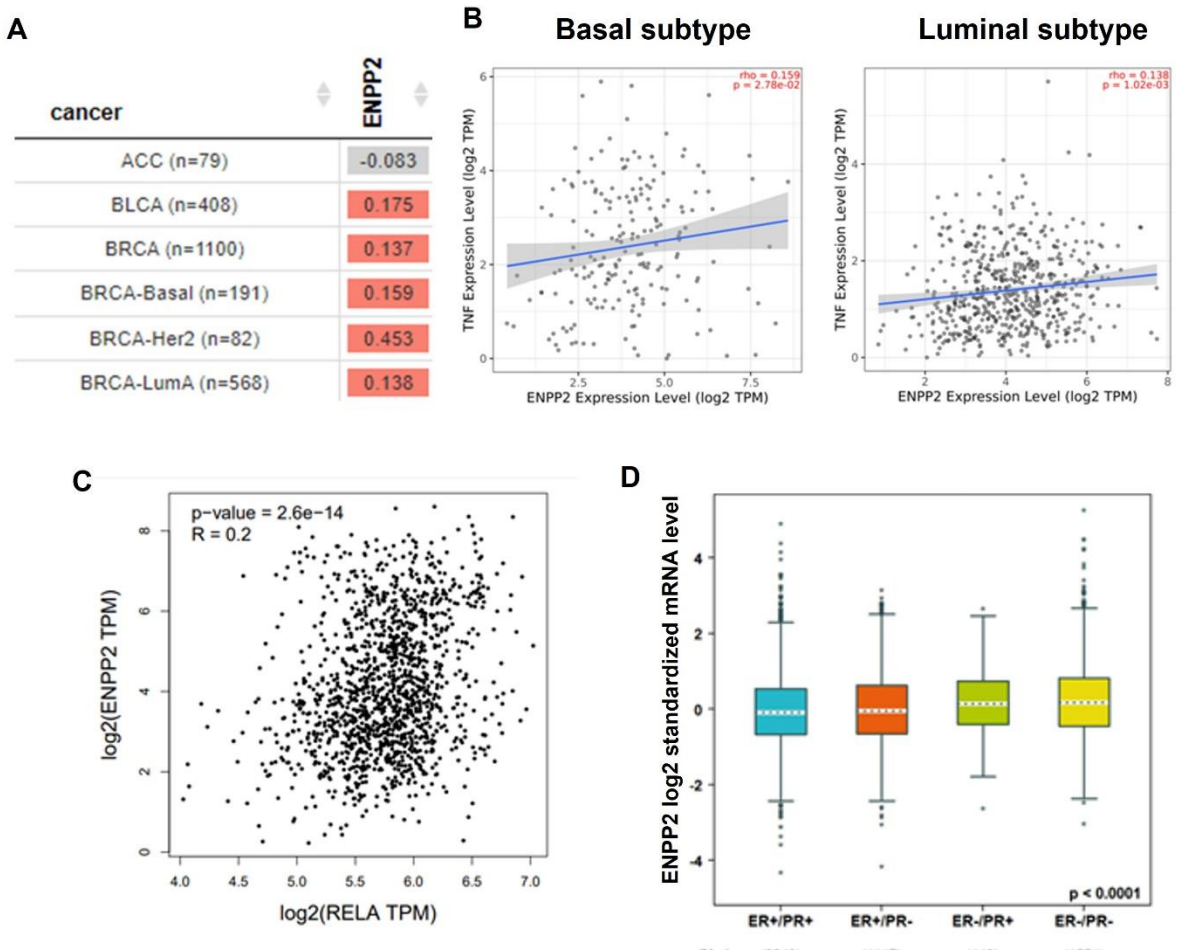


Figure 27: Positive correlations among TNF-α, RELA and ATX in breast cancer (A&B) Positive correlation between TNF-α and different subtypes of breast cancer patients **(C)** Positive correlation between RELA and ATX in breast cancer patients **(D)** Expression of *ENPP2 gene (ATX)* in breast cancer patients with varying ER/PR status

4.4.5 TNF-α induced NF-κB and ATX activity are correlated in breast cancer

The role of TNF-α in regulating the ATX expression in breast cancer was further validated in the tumor tissue of Infliximab treated mice. As established earlier, Infliximab decrease TNF-α-induced NF-κB activity and so the study evaluated TNF-α-induced ATX expression in these conditions. A significant decrease was observed in the ATX staining by IHC using specific antibody for ATX in the Infliximab treated group (Figure 28A). In addition, ATX activity and ATX mRNA in the primary tumor tissue were also decreased in Infliximab treated mice (Figure 28B,C). Thus, these results show that blocking TNF-α activity *in vivo* decreased the expression of ATX in the primary breast tumors.

Next, the effects of blocking ATX activity in mice was determined using the ATX inhibitor, IOA-289 as described previously (34). A significant decrease was observed in the RELA expression in stumors of IOA-289-treated mice compared to the controls (Figure 28D). Furthermore, the study investigated the role TNF-α-induced NF-κB in regulating ATX expression by using parthenolide (NF-κB inhibitor) *in vitro.* 4T1 cells showed upregulation in ATX expression on treating with TNF-α for 24h (Figure 28E). ATX expression in 4T1 breast cancer cells in the presence of TNF-α was decreased when treated with parthenolide (an NF-κB inhibitor) (Figure 28F).

Further the study analysed the clone forming ability of 4T1 cells in different treatment conditions. TNF-α increased the number of clones formed by 4T1 cells compared to control (Figure 28 G-H). TNF-α in combination with parthenolide showed rescue when compared to parthenolide alone. The clone forming ability was not affected significantly by LPA alone but there was an increase in combination with TNF-α. This appeared to be decreased by parthenolide, but it did not reach the level of significance (p value=0.084). To analyse these results further, the average colony sizes was measured and observed an increase in presence of TNF-α, LPA or the combination compared to the untreated control (Figure 28I). The average colony size decreased in presence of parthenolide and was partially rescued by addition of TNF-α and LPA or the combination. These results strengthen our hypothesis that activation of NF-κB can increase the expression of ATX in breast cancer and thereby increase the proliferation and metastatic potentials. Together, these results show that TNF-α induced NF-κB activation increases the limited ATX expression in breast cancer cells and LPA production increases clonogenicity.

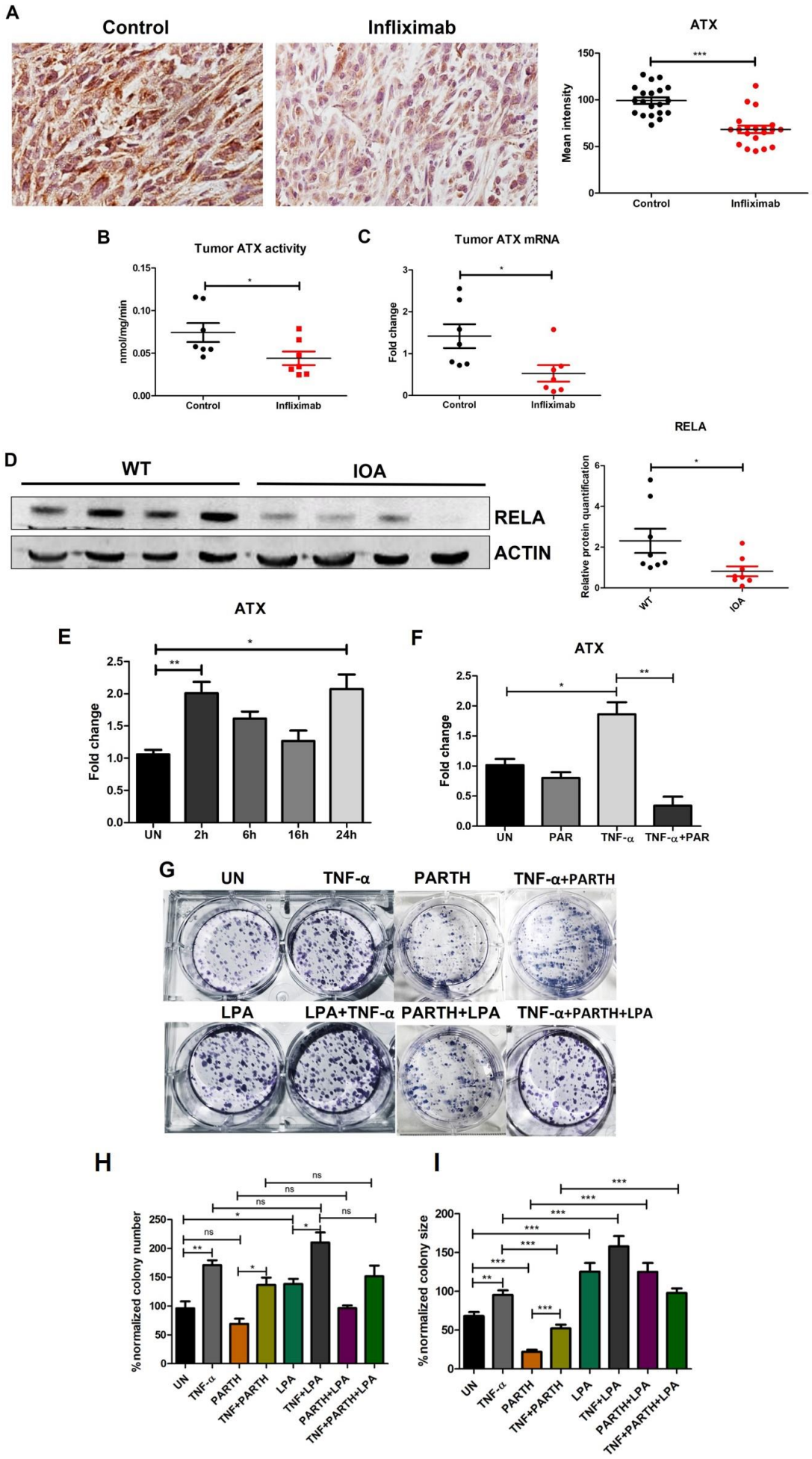

A
Control
Infliximab
ATX
Mean intensity
Control
Infliximab
B
Tumor ATX activity
nmol/mg/min
Control
Infliximab
C
Tumor ATX mRNA
Fold change
Control
Infliximab
D
WT
IOA
RELA
ACTIN
RELA
Relative protein quantification
WT
IOA
E
ATX
Fold change
UN
2h
6h
16h
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Figure 28: ATX and TNF-α-induced NF-κB activity are correlated in breast cancer and effects clone forming ability (**A**) IHC staining using an ATX-specific antibody in primary tumors from control and Infliximab treated mice (**B**)Tumor ATX activity in control and Infliximab treated mice. (**C**)Comparison of ATX mRNA expression in Infliximab and control mice by qRT-PCR analysis. (**D**)RELA levels in E0771 tumors of C57BL/c mice treated with the ATX inhibitor, IOA-289, versus controls by western blotting. (**E**) Expression of ATX in 4T1 cells treated with TNF-α (10ng/ml) for different time points by qRT-PCR. (**F**) Expression of ATX in 4T1 cells treated with TNF-α (10 ng/ml) for 24h and parthenolide (5 μM) for 4h by qRT-PCR. (**G**) Colony size of 4T1 cells treated with TNF-α (10 ng/ml), Parth (1 μM) and LPA (10 μM) was measured by ImageJ. (**H&I**) Analysis of colony number and size for colony forming assay(n=3)

4.4.6 Discussion:

Inflammation inducing factors in the TME can cause adverse effects on the metastasis and treatment of breast cancers [274, 275]. TNF-α has a dual role in breast cancer and its effect are mainly regulated depending on the concentrations in the tumor and the TME and is responsible for breast cancer cell survival or cell death [276].TNF-α can be produced both by the breast cancer cells along with immune cells in the TME, which regulates diverse processes such as cell-cell communication, proliferation, differentiation, metastasis and cell death [277]. TNF-α-induced inflammation is associated with disease severity and a higher rate of recurrence in breast cancer[278]. Biopsy samples of breast tumors show increased TNF-α expression and this has been associated with worse prognosis [279]. Despite intensive research in TNF-α-induced effects in breast cancer biology, details of the molecular mechanisms of TNF-α action remain largely unexplored. The current study report inhibition of TNF-α signalling in syngeneic mice model of breast cancer using Infliximab inhibited metastasis specifically to lung by inhibiting NF-κB and LPA-ATX signaling.

The study used Infliximab, a chimeric human-mouse monoclonal antibody, which consist of human IgG1 Fc region fused with murine Fv region, against TNF-α. The antibody binds specifically to TNF-α and prevents interaction with TNF-α receptors. Earlier, Infliximab-based studies showed a remarkable decrease in the metastasis of osteosarcoma cells to lungs. Related studies in advance cancer showed that Infliximab was well tolerated with few toxic effects, and it decreased inflammatory factors including CCL2, IL-6 and serum CRP. Our results established that Infliximab-treated mice showed marginally reduced breast tumor weight compared to control mice. Our major finding was a ~60% reduction in breast cancer metastasis to lungs in the Infliximab treated group. Moreover, a significant decrease was observed in

metastatic and proliferation markers such as tumor Vimentin and Ki-67 respectively, in the Infliximab-treated group. Ki-67 expression has been well corelated with breast cancer subtypes and relapse in patients [280]. Studies related to Vimentin in breast cancer cells have considered it as a new target for therapeutics and have shown it to be associated with recurrence of Triple Negative Breast Cancer (TNBC) [281]. Overall, these findings together with our present study validate that blocking TNF-α signaling can decrease breast tumor growth and have an even more marked effect in decreasing metastasis.

The correlation among inflammation, cytokine and chemokines produced in the tumor to promote growth and metastasis has been studied extensively [282, 283]. Crosstalk among different cell types in TME shows that cytokine like IL-1β produced by activated fibroblasts create a tumor promoting environment for ER positive breast cancer cell growth [284]. IL-6 expression in 249 patient serum samples was significantly increased and this was associated with recurrence risk of breast cancer [37]. TNF-α-induced NF-κB activation regulates the expression of multiple inflammation factors, which leads to favourable cytokine milieu in the TME required for tumor progression. A marked increase occurs in the expression of inflammatory cytokines such as IL-6, IL-8 and TGF-β upon TNF-α stimulation in TNBC. Such events also trigger expression of EMT markers and increase metastatic potentials [285]. The study investigated the effects of Infliximab on the concentrations of 32 cytokines, chemokines and growth factors in plasma and primary tumors. A decrease was observed in the TNF-α, IL-4, MCP-1 and an increase in the IFNγ levels in the plasma of Infliximab group compared to control. Patient studies involving 768 individuals with varying genotypes have shown that TNF-α is a critical player in tumor progression and distant metastasis, especially in TNBC [286]. Decreased serum levels of TNF-α were also associated with anti-tumor effects in an MCF-7-derived breast cancer in a mouse model treated with the opiate, tramadol, which is associated with reduced mortality in patient [287]. TNF-α gene knockout studies in TNBC cell lines show inhibited cell proliferation and induced apoptosis [288]. IL-4 signaling was identified to trigger tumor-associated macrophages and enhance invasion in breast cancer [289]. Dutta et al. showed that MCP-1 is increased in TNBC cell lines compared to ER-positive MCF-7 cells and explored its role in cell invasion and increasing EMT markers, like N-cadherin and Vimentin [290]. IFN-γ was shown to have anti-tumor effects by decreasing cancer stem cell numbers in 4T1 mouse model of breast cancer [291].

Furthermore, IL-4 and IL-12p40 were also decreased in the breast tumors by Infliximab treatment. Human breast cancer cells produce IL-12p40 as a mechanism to suppress the anti-tumor immune responses and protect the tumor from the immune system [292]. Other studies showed that decreasing inflammatory cytokines has a promising role in controlling breast cancer invasion and metastasis [293]. This profiling identified potential candidates that can contribute in the invasiveness and metastatic potentials of the breast cancer cells and these are mainly regulated by the NF-κB pathway.

Next, experimentally it verified the activation of NF-κB pathway in the primary breast tumors, which is the major pathway activated upon TNF-α stimulation. NF-κB activation is a typical phenomenon, which is detected in breast cancer [294]. The genes that are altered by NF-κB activation are often the prime source behind disease aggressiveness and poor prognosis [295, 296]. The study further analysed NF-κB responsive genes, such as those for *TNF-α, IL-6, IL-18 and RELA*, which showed reduced mRNA levels in Infliximab-treated mice. IL-6 has been identified in promoting tumorigenesis and metastasis in breast cancer. It is considered as a key cytokine effecting STAT3 phosphorylation in many cancers and it is associated with poor prognosis [297, 298]. Engineered bispecific antibodies that target both IL-6 and IL-8 decrease the migratory potentials of metastatic TNBC [299]. Serum IL-18 levels in patients with gastric cancer and skin cutaneous melanoma have been cor-related with cell migration and malignancy [80-81]. Studies have also shown that IL-18 plays an important role in inducing breast cancer cell migration by activating p38 MAPK pathway and downregulation of claudin-12 [82].

Further, the study validated the involvement of NF-κB activation by the nuclear translocation of p65. RELA translocate to the nucleus to activate NF-κB signaling. Kanzaki et al. showed that inhibiting the nuclear translocation of RELA decreases the growth of TNBC [90]. Ubiquitination of p65 (RELA) and its degradation by E3 ligase FBXW2, leads to suppressed breast cancer stemness and tumorigenesis [266]. IHC staining revealed a reduced colocalization of p65 with the nucleus in the Infliximab-treated mice. Furthermore, reduced TNF-α in the plasma of Infliximab-treated mice was associated with a decreased phosphorylated of IκBα, which on ubiquitination and proteosomal degradation activates the NF-κB pathway. These results establish that NF-κB activation is decreased by Infliximab-treated mice compared to the controls.

The cross-talk among different pathways in breast cancer and their cumulative effects on cell death or survival needs extensive investigation. Therefore, the study focussed on the ATX-LPA-inflammatory cycle, which is drives breast tumor growth and metastasis. ATX expression

is stimulated by inflammatory cytokines [107, 111]. ATX secretion is stimulated after tissue injury and promotes wound healing by increasing cell migration and division. ATX does this by producing lysophosphatidate (LPA), which signals through six G-protein coupled receptors. Inflammation during wound healing increases the secretion of ATX and consequent LPA production, which then activates NF-κB and stimulates the production of more inflammatory cytokines in a feedforward cycle [113]. This inflammatory cycle is resolved under normal circumstances, but chronic activation of the ATX-LPA-inflammatory axis in cancers leads to enhanced tumor growth and especially metastasis [300]. Increased LPA signaling has been associated with metastatic tumors and breast tumorigenesis [301]. Previous studies from our group have emphasized the importance of ATX in the TME and established that inhibiting ATX can reduce breast cancer metastasis to lungs [302]. Basal and TNF-α-induced NF-κB activation and ATX expression also plays important role in liver tumorigenesis [107]. Thus, it was important to dissect the role of TNF-α-induced inflammation in ATX-LPA signaling in breast cancer.

Firstly, cancer databases were explored and it was established that TNF-α, RELA and ATX in breast tumors showed positive correlations in patients. Increased expression of the *ENPP2 (ATX)* gene was observed in highly metastatic TNBC patients compared to less metastatic ones. These results strengthened our hypothesis and laid foundation for further studies. ATX-LPA signaling is driven by an inflammatory cycle implying that decreasing inflammation should attenuate ATX-LPA signaling. Previous studies have showed this effect by treating mice with dexamethasone and establishing that this decreased ATX-depended LPA signaling [303] and the effects of radiation in activating this pathway [304]. Dexamethasone is a very effective anti-inflammatory, but it has limited application in cancer therapy because of side-effects and immune-suppressive properties. Therefore, choice of Infliximab for the present studies is much more relevant to cancer therapy.

As expected, a decrease in the ATX staining in tumors of Infliximab-treated mice was observed together with decreased ATX activity and mRNA levels. This was accompanied by decreased lung metastasis. Previous studies also showed that down-regulating endogenous ATX in 4T1 cells reduces osteolytic bone metastasis in BALB/c mice [301].

To further establish the link between ATX and NF-κB, the expression of RELA after ATX inhibition was measured in mice with IOA-289, which is now in a Phase 1B trial for treating pancreatic cancer [72]. Interestingly, IOA-289 decreased RELA expression in breast tumors.

This observation establishes an important link between the TNF-induced NF-κB pathway and ATX expression in breast cancer. It also provides a further explanation for why IOA-289 decreases the growth of breast tumors and metastasis in two mouse models [34, 69].

The study also established that TNF-α increases ATX expression in 4T1 breast cancer cell line and increases the clone forming ability, which depends on the activation of NF-κB. It should be noted that ATX expression is low in breast cancer cells and that other tumor cells, including fibroblasts, leukocytes and endothelial cells, are probably much more important in the ATX production that drives breast tumor growth and metastasis [66]. Also, TNF-α and LPA in the present work increased size of clones formed by 4T1 breast cancer cells.

Taken together, this study highlights the role of TNF-α-induced NF-κB activation in regulating inflammatory signaling and explores new molecular insights that can attenuate the invasiveness and metastatic properties of breast tumors. In particular, the study showed that the effects of TNF-α induced inflammation are mediated partly by increased ATX expression and LPA signaling, which increases the metastatic potential of tumors. This work provides an increased understanding of how to regulate the ATX-LPA-inflammatory cycle as a novel future treatment for improving the outcomes for breast cancer patients with developing combinatorial treatment using infliximab (decreasing effective TNF concentration) and ATX-LPA inhibitors.