PhD thesis plan

MOLECULAR REGULATION OF LYMPHANGIOLEIOMYOMATOSIS

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2019

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1. ABBREVIATIONS

3BP	3-bromopyruvate
4E-BP1	4E-binding protein
AKT	Protein kinase B
ALDH1A1	Alcohol dehydrogenase
AMPK	AMP-activated protein kinase
a-SMA	Alpha smooth muscle actin
ATF2	Activating transcription factor 2
ATG13	Autophagy-related gene
BCO1/2	Beta-carotene dehydrogenase 1/2
BIM	Bcl-2 like protein
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein
CaMKII	Ca-Calmodulin kinase II
CCR9	C-C motif chemokine receptor
cFOS	FOS proto-oncogene
COPD	Chronic obstructive pulmonary disease
CREB	cAMP response element binding
CXCL16	C-X-C motif chemokine ligand
d4E-BP	Eif4e binding protein
Deptor	DEP domain-containing mTOR-interacting protein
DKK	Dickkopf
DVL	Dishevelled
eEF2K	Eukaryotic elongation factor 2 kinase
EGF	Epidermal growth factor
ERa	Estrogen receptor alpha
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
ESRRγ	Estrogen related receptor gamma
Factor VIII	Von Willebrand Factor
FIP200	Focal adhesion kinase family-interacting protein
Fzd	Frizzled receptor
GAP	GTPase activating protein
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GSK3	Glycogen synthase kinase
IGF	Insulin growth factor
IL	Interleukin
INF-γ	Interferon gamma
IRS1	Insulin receptor substrate 1
LAM	Lymphangioleiomyomatosis
LPR5/6	Low-density receptor-related protein 5/6
MAM	Mitochondria-associated ER membrane
MAPK	Mitogen activated protein kinase
mLST8	Mammalian lethal with Sec13 protein
MMP	Matrix metalloproteinase
mSIN1	Mammalian stress-activated protein kinase interacting protein
mTOR	Mammalian or mechanistic target of rapamycin
NR5A2	Nuclear receptor subfamily 5 group A member 2
NRF1	Nuclear respiratory factor 1
PCP	Planar cell polarity
PDCD4	Programmed cell death 4

PDGFR	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PFK	Phospho-fructo kinase
PGC1	Peroxisome proliferator-activated receptor-γ coactivator 1
PGR	Progesterone receptor
PI3K	Kinases phosphoinositide 3 kinase
PKC	Protein kinase C
PKM2	M2 isoform of pyruvate kinase
PML	Promyelocytic leukaemia protein
PPARγ	Peroxisome proliferator-activated receptor-γ
PPARGC1β	PPAR gamma coactivator 1 beta
PR	Progesterone receptors
PRAS 40	Proline-rich AKt1 substrate
Protor-1	Protein observed with Rictor-1
RA	Retinoic acid
Raptor	Regulatory-associated protein of mTOR
RARβ	Retinoic acid receptor beta
RDH	Retinol dehydrogenase
REDD1	Regulation of DNA damage response 1
Rheb	Ras homolog enriched in brain
RISK1	Retrogradely transported kinase
S6K1	Ribosomal protein S6 kinase beta
SKAR	S6K1 aly/REF-like target
SLC7A5	Solute carrier family 7 member 5
SREBP1	Sterol regulatory element binding protein 1
TCF/LEF	T-cell factor/lymphoid enhancer factor
TFAM	Mitochondrial transcription factor A
TNF-a	Tumour necrosis factor alpha
TrxR	Glutathione reductases and thioredoxin reductases
TSC1	Hamartin
TSC2	Tuberin
TSP1	Thrombospondin 1
ULK1	Unc-51-like kinase 1
VEGF/VEGFR	Vascular endothelial growth factor/ Vascular endothelial growth Receptor
YY1	Transcription factor yin-yang 1

2. INTRODUCTION

2.1. Epidemiology of LAM

Lymphangioleiomyomatosis (LAM) is a rare, slowly progressing, fatal systemic disease that affects mainly young women [1]. LAM is defined by the overgrowth of atypical smooth muscle (SMC)-like cells known as LAM cells in the lungs, lymphatics and soft organs [2]. Excess growth of atypical SMC-like cells leads to cyst formation within the lungs resulting in pneumothoraces and lung collapse. LAM was formerly considered as a benign disease, but recently such cyst forming cellular growth has been reclassified as slow-growth-rate metastasizing neoplasm [2]. The suffering of LAM patients increases gradually as the disease progresses. LAM is categorized as a rare disease worldwide with an incidence rate of 1-9/1 000 000. In Europe, numbers are much higher where the sporadic form of LAM affects 1/500,000-1/125,000 women. Also, LAM characteristic mutation of the tuberous sclerosis (TSC) gene is present in 1/6000 births in Europe. 30-40% of people carrying TSC mutation develop LAM in their adulthood [3]. With the new and more sensitive diagnostic methods, numbers are climbing worldwide and increase significantly in Europe. Apart from TSC mutation no other genetic mutations have been reported yet to explain the higher numbers in Europe. The majority (86%) of patients need lung transplantation within 10 years after diagnosis. All LAM patients experience a gradually decreasing quality of life until even basic activities become impossible [4].

2.2. Clinical features, mutations and diagnostic markers

Most of LAM patients present to the clinic with shortness of breath, chest tightness, collection of air in the pleural space (pneumothorax) and excess fluid between the layers of the pleura (pleural effusion) [5]. These symptoms are not specific to LAM, therefore the disease is often misdiagnosed as asthma or chronic obstructive pulmonary disease (COPD). This misdiagnosis leads to failed treatment and faster disease progression as asthma and COPD medications have limited effects on the symptoms [3]. Diagnosis can only be made if vascular endothelial growth factor D (VEGF-D) (above 800 pg/ml) and matrix metalloproteinase (MMP) plasma levels are measured and lung CT scan confirms the diagnosis [6]. In many cases, a tissue biopsy is needed to confirm LAM, especially, in the absence of family history of the disease. The tissue biopsy is taken from a nodule of cyst forming atypical SMC-like cells and the surrounding lymphatic endothelial cells. Using video-assisted thoracoscopy, the obtained nodule is subjected to histology. Immunohistochemical staining of atypical SMC-like cells shows a similar staining

pattern to normal SMC-s for various markers including alpha smooth muscle actin (*a*-SMA), vimentin and desmin [7]. However, in contrast to normal SMC-s, LAM cells are also positive for melanocytic marker MelnaA (HMB-45) and ribosomal protein S6 (PS6) [8] which are in the presence of SMA make a rather distinctive staining combination as LAM diagnostic markers [9]. Also, changes in mammalian or mechanistic target of rapamycin (mTOR) activity [10] and TSC1/2 mutations [11] compared to normal cellular activity are characteristic to LAM and are useful markers (see in detail below).

2.3. The role of estrogen in LAM pathogenesis

As mentioned above, LAM occurs almost exclusively in young women. A few cases have been reported in men, where the loss of TSC2 gene occurred spontaneously [12]. It seems that sex hormones have a role in LAM development and progression. Apart from the almost exclusively female occurrence, the symptoms of LAM are aggravated during pregnancy and estrogen supplement therapy [13]. Estrogen has been linked to many cancers in women, especially breast cancer, but the full mechanism is still poorly understood [14]. *In vitro* studies support the role of sex hormones in LAM development and in progression as well [15]. The vast majority of cyst biopsies express high levels of estrogen receptor alpha (ERα) [9] and progesterone receptors (PR) [9]. Previous studies have shown that ERα is an effective mediator of proliferation induced by estrogen in breast and uterus cancer, where treatment of cells *in vitro* with sex hormones promote ERα expression [9]. ERα acts via the mitogen activated protein kinase (MAPK) pathway and increases proliferation of LAM cells, but the role of progesterone in the progression of LAM is still unclear [9].

2.4. The origin of LAM cells

The origin of characteristic LAM cells is still unknown, despite the indicative presence of various tissue markers. As mentioned above, reactivity to α-SMA and HMB45, altered mTOR-S6P activity, and TSC1/2 mutations [8] are all characteristic to LAM cells. Expression of the melanocytic marker HMB45 has raised a theory that LAM cells might potentially develop in the neural crest [8]. Certainly, cyst formation in tissues that originate from the neural crest lineage support such theory [18]. Another hypothesis places the uterus as the origin of LAM cells. This theory is supported by the high levels of ER and PR expression in LAM cells showing 30 % correlation with angiomyolipomas, perivascular epithelioid cell tumours (PEComas) and mesenchymal tumours most commonly found in the uterus [19]. Unfortunately, even if the origin of the LAM cells is

going to be resolved, the theory could not explain how the cyst migrates and metastasizes to the lung [20]. An observation of lymphatic endothelial cells surrounding the LAM cluster raised a new theory involving the lymphatic system [5]. High expression levels of vascular endothelial growth factor C (VEGF-C), VEGF-D and VEGFR-3 proves that vascularization and the lymphatic system are important in the process [5].

Kumsaka et al. summarized the theory that LAM originates in an organ rich in SMCs e.g. kidney or uterus, and upon the formation of the cyst, some clusters start to bud from the endothelial tissues and enter the lymphatic system. This is the transition phase and at that stage LAM clusters start to involve the surrounding lymphatic tissue and increase VEGF expression levels. Via the circulation the clusters reach the thoracic duct and start invading and infiltrating the lung tissue. Finally, they are settling in the alveolar region. Uncontrolled growth of LAM clusters and increased cyst size progressively block lung function and patients present with various asthma- or COPD-like symptoms at the clinic. Later, uncontrolled cyst growth results in pneumothoraces and hospitalization [22].

2.5. Molecular background of LAM (upstream signals of mTOR)

mTOR is effectively regulated by many signals (Figure 1 and 2). One of the main regulators of such signals involve a dimer of two proteins hamartin and tuberin that are associated with both TSC1 and TSC2. Hamartin (129.7 kDa) acts as a scaffold protein by stabilizing the function of tuberin. Tuberin (200.6 kDa) has a GTPase activating protein (GAP) domain at the carboxylic terminus. When hamartin stabilizes tuberin the GAP domain blocks the activity of Rheb (Ras homology enriched in brain that belongs to the Ras superfamily of monomeric GTPases). Rheb activates mTOR, the catalytic component of mTORC1 (Figure 1). TSC1/2 are the main regulatory proteins of mTORC1, therefore any changes in TSC1/2 mechanism results in up-regulation of mTOR activity and results in increased proliferation of cells leading to proliferative diseases like LAM [23].

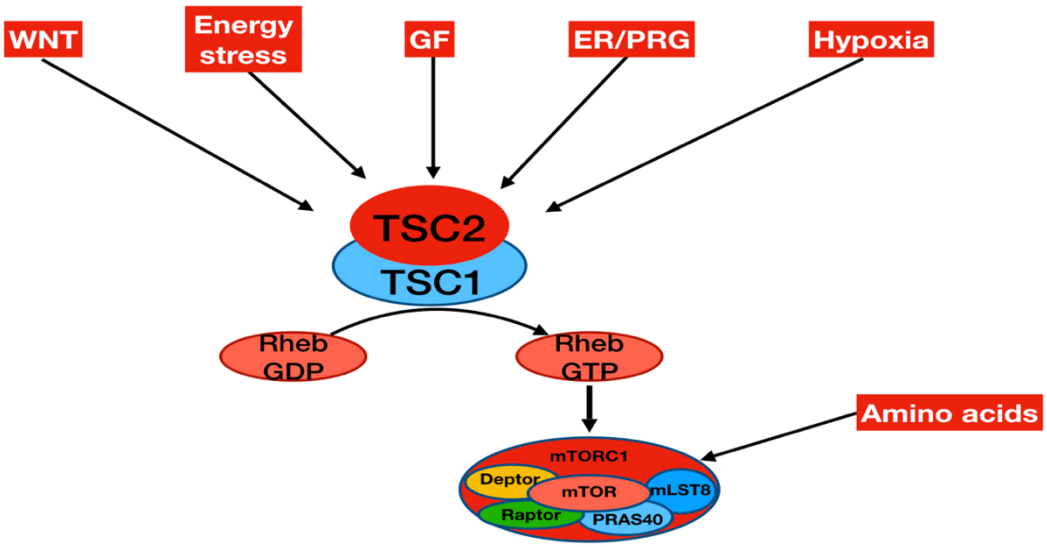


Figure 1. Upstream signals affecting the mTORC1 signalling pathway.

As mentioned above, the **mTORC1** pathway has many up-stream regulatory molecules and pathways including growth factors [24], energy depletion mechanisms, changes in oxygen levels, amino acids (leucine and arginine), altered levels of inflammatory cytokines, estrogen [24] etc. One of the main mTORC1 regulatory pathways is the evolutionarily conserved Wnt signalling pathway [25]. Currently, the regulatory factors of the mTOR pathway are under intense investigation as they might provide novel and potentially more effective therapeutic targets. *Growth factors (GF),* for example, either protein factors or steroid hormones can modulate LAM progression. Earlier studies show a correlation between platelet-derived growth factor (PDGFR), epidermal growth factor (EGF) and its receptor EGFR and mTOR signalling pathways via the activation of downstream kinases including phosphoinositide 3 kinase (PI3K), protein kinase B (AKT) and mTORC1. Most GFs act on mTORC1 via increasing the phosphorylation of TSC1/2 by AKT yet in some cases AKT can also act directly on proline-rich AKt1 substrate 40 kDa (PRAS40) leading to dissociation of mTORC1 [23]. Also, Ras/ Extracellular signal-regulated protein kinases 1 and 2 (ERK1,2), retrogradely transported kinase 1 (RISK1) form another pathway that GFs can use to inhibit TSC1/2 [26]. Insulin and insulin like growth factor 1 (IGF-1) (a hormone similar in molecular structure to insulin) both play an important role in anabolic activity and growth. Upon binding of insulin to its cell surface receptor the tyrosine kinase recruits insulin receptor substrate 1 (IRS1) leading to

activation of PI3K and in a following step to activation of AKT and inhibition of TSC1/2 activity. The above signalling steps promote mTORC1 activity and its downstream protein, ribosomal protein S6 kinase beta 1 (S6K1) activation [27]. S6K1 act as a negative feedback to IRS1 resulting in impaired insulin stimulated glucose uptake [27]. *Changes in energy and oxygen levels* can also affect mTORC1 activity. AMP-activated protein kinase (AMPK) acts as a sensor of low cellular energy levels [28]. Upon sensing a decrease in energy levels, AMPK phosphorylates TSC2 leading to increased GAP activity. TSC2 blocks Rheb resulting in the reduction of mTORC1 activity. In the case of energy depletion AMPK can act directly on Raptor to reduce mTORC1 activity [39]. Oxygen levels can also affect mTOR activity. Hypoxia can drive mTORC1 activation via transcriptional regulation of DNA damage response 1 (REDD1). REDD1 interrupts TSC2 interaction with inhibitory protein 14-3-3. As a result, TSC2 will directly affect mTORC1 activity. The other two factors that affect mTOR activity during hypoxia are the tumour suppressor BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) [30] and the tumour suppressor promyelocytic leukaemia protein (PML) [31]. They are both affecting the mTOR pathway by blocking the interaction between mTORC1 and Rheb [31]. *Amino acids* like leucine and arginine can act directly on mTORC1. This process is independent from TSC1/2 and is essential for mTORC1 activation. Leucine enters the cells in exchange for glutamine via the heterodimeric solute carrier family 7 member 5 (SLC7A5) [32]. Higher concentration of Leucine in the cytosol will stimulate Rag-GTPase activation that will stimulate mTORC1 activation [43]. Activated mTORC1 by Rag-GTPase will facilitate the translocation of mTORC1 to the perinuclear region of the cell via linking Rag to Raptor [32]. *Sex hormones* also have a role in the development and progression of many diseases that occur predominantly in women [34]. ERα is an effective mediator of proliferation induced by estrogen in breast and uterus cancer, and treating cells in-vitro with sex hormones promote ERα expression. ERα acts via the MAPK-Fos related antigen 1 (Fra1) pathway and increases proliferation of LAM cells [34]. Another study proposed an alternative mechanism that in the presence of estrogen ERα binds to raptor via the TOS motif [35]. As a result, mTORC1 is recruited to the nucleus where mTOR phosphorylates ERα leading to its activation and resulting in upregulation of estrogen dependent gene transcription [35]. Progesterone also affects the mTOR pathway but the mechanism is not clear yet [36]. *Retinoic acid* (RA) is an important metabolite of retinol (vitamin A). Retinoic acid plays an important role in cell development, differentiation and cellular proliferation. Not surprisingly, RA affects the growth of different types of cancer cells in breast, prostate and lung cancer as well as

melanoma [37]. Previous studies have revealed crosstalk amongst mTORC1, estrogen and RA signalling [38] explaining the importance of RA in proliferative diseases [39]. mTORC1 activation has also been shown to promote the expression of *peroxisome proliferator-activated receptor-γ* coactivator 1 (PGC1) [40], a coactivator of nuclear receptors like ERs and ERRs [41]. Estrogen and RA both signal via nuclear receptors and are known for their opposing effect on cancer cell growth and proliferation [42]. Treatment of T cells with RA induces C-C motif chemokine receptor 9 (CCR9) expression which process is also dependent on mTOR activity [43]. However, one of the most complex mTOR regulator is the *Wnt signalling* pathway that affects mTOR activity at several levels.

2.6. Wnt signalling

The role of Wnt signalling both in development and carcinogenesis is crucial and, despite intense study of the pathway and its specific molecular elements in the past decades, it is still not fully understood. One of the problems is that the mammalian Wnt family consist of 19 secreted glycoproteins that are extremely promiscuous when it comes to receptor binding [44]. Traditionally, there are three main Wnt signalling pathways, the β-catenin dependent classical or canonical, and two non-canonical pathways including the Ca^{2+} dependent and the cJun N terminal kinase dependent planar cell polarity (PCP) pathways [45]. Some Wnt ligands including Wnt1, Wnt3, Wnt3a, Wnt7a, Wnt7b and Wnt8 mostly activate the β-catenin dependent canonical pathway [44], while Wnt5a, Wnt5b, Wnt4 and Wnt11 are preferentially activators of the non-canonical Wnt signalling pathways (Figure 2) [55].

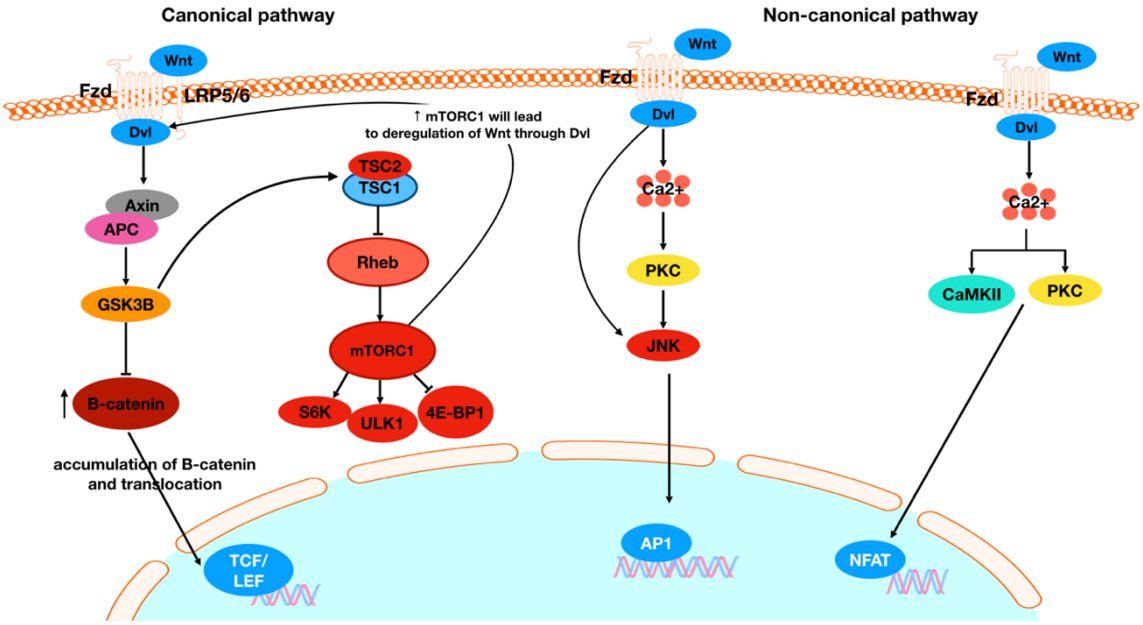


Figure 2. Wnt signalling pathways. Schematic illustration of canonical and non-canonical pathways and the signalling crosstalk with the mTOR pathway [44].

The above described Wnt ligands can choose to bind one of the ten seven-transmembrane domain receptors, frizzleds (Fzd). In the presence of canonical Wnt, Fzd and a single-membrane-spanning low-density receptor-related protein 5/6 (LPR5/6) form a complex, then Fzd recruits, phosphorylates and activates dishevelled (Dvl) [46]. Activated Dvl will inhibit GSK3β resulting in reduced phosphorylation of the enzyme and that consequently leads to inhibited proteolytic destruction of β-catenin [47]. Accumulated β-catenin in the cytosol then translocates to the nucleus where it forms a transcription complex with the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors [47]. The non-canonical Wnt signalling pathways differ from the canonical pathway by the type of G-proteins they require and the lack of β-catenin in the activation of the Ca^{2+} and the PCP pathways [48]. In the Ca^{2+} dependent Wnt pathway Ca^{2+} activates protein kinase C (PKC) and Ca-Calmodulin kinase II (CaMKII) and both trigger gene transcription using the nuclear factor of activated T-cells (NFAT) transcription factor family. NFAT regulates the transcription of many genes that play important roles in immunological reactions. The target genes include interleukin (IL)-2, IL-4, interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α). Meanwhile, the PCP pathway activates the AP1 transcription complex that is formed from proto-oncogenes cJun, JunB, JunD, FOS proto-

oncogene (cFos), FBJ murine osteosarcoma viral oncogene homolog B (FosB), Fra1, Fra2, activating transcription factor 2 (ATF2), and cAMP response element binding (CREB) proteins [44]. Upon activation of the AP1 complex, transcription of many genes including cyclin D1, matrix metalloproteinase 3 (MMP-3), Bcl-2 like protein 11 (Bim) and granulocyte macrophage colony stimulating factor (GMCSF) are initiated [49]. Apart from being important in lung development (Wnt11, Wnt7b) [50], dysfunction of the Wnt pathways is also linked to lung cancer (e.g. loss of Wnt7a [51], elevated Wnt1 and Wnt2 [52], Wnt5a [53]). Since the Wnt signalling pathway plays an important role in tissue maintenance, it falls under complex regulation involving a multitude of extra- and intracellular molecules including Dickkopf Wnt signalling pathway inhibitor (Dkk-s), secreted frizzled related protein (sFRP)-s, Wnt inhibitory factor (WIF), cytosolic catenin β interacting protein (ICAT), Naked cuticle homolog (Nkd) and nuclear SRY related HMG box (Sox17) [44].

Due to its complexity, it is not surprising that Wnt signalling has a connection to mTOR. Some aspects of this interaction has been studied in depth including the ability of mTORC1 to suppress the canonical Wnt pathway via modulation of Dvl [54] and GSK3β activity [55].

mTOR regulation by Wnt signalling in neoplasms

Wnt/β-catenin signalling increases aerobic glycolysis via suppression of mitochondrial respiration by reducing cyclooxygenase (COX) transcription [56]. Wnt5b controls the expression of the OXPHOS-related cytochrome c1 and the ATP synthase γ subunit expression [57]. Canonical Wnt signalling can also promote aerobic glycolysis via increased expression of pyruvate dehydrogenase kinase 1 (PDK1) that inhibits mitochondrion-bound pyruvate dehydrogenase (PDH) and decreases pyruvate oxidation, resulting in increased pyruvate conversion to lactate in the cytosol [58]. The upregulated lactate transport facilitates angiogenesis. Both derailed canonical and non-canonical Wnt signalling is linked to VEGF expression and increased angiogenesis in lung cancer (LC) [59]. Additionally, one of the canonical Wnt pathway targets the proto-oncogene c-Myc apart from controlling the cell cycle, regulates gene transcription of glycolysis, nucleotide synthesis, lipid synthesis, glutaminolysis, mitochondrial bioenergetics, autophagy and production of reactive oxygen species. Oxidative stress can activate canonical Wnt signalling in a range of cell types upstream of β-catenin at the level of Dvl and increases TCF-dependent proliferation [60][61]. Additionally, β-catenin-mediated c-Myc

expression affects expression of glucose transporter 1 (GLUT-1), LDH, and the M2 isoform of pyruvate kinase (PKM2) [43]. PKM2 is a pleiotropic protein and can also function as a transcriptional coactivator. Nuclear translocation of PKM2 facilitates its interaction with β-catenin downstream of epidermal growth factor (EGF) signalling and elicits β-catenin-induced transcriptional changes. There is also cross talk between the canonical Wnt and EGF signalling pathways, as EGF–PKM2–β-catenin signalling results in increased expression of dickkopf 1 (DKK-1), a canonical Wnt pathway inhibitor [44]. In addition, nutrient-sensing AMPK signalling pathways also regulate canonical Wnt signalling. AMPK is an energy sensor of intracellular AMP/ATP ratios [25]. Activated AMPK can inhibit Wnt/β-catenin signalling via reduction of Dvl activity. However, Wnt signalling can also alter cancer cell metabolism in a β-catenin independent manner. The AKT-mTOR signalling pathway provides potent control over metabolic reprogramming during tumorigenesis by regulating nutrient uptake and allocating carbon and nitrogen to anabolic pathways. mTOR signalling increases aerobic glycolysis in cancer cells by increasing GLUT expression and stimulating glycolytic enzyme activity [62]. mTOR is regulated by AKT-mediated inactivation of the mTOR upstream regulator TSC, where TSC frees the GTPase Rheb to directly activate mTORC1. Wnt1 as well as Wnt3a can induce mTOR signalling via GSK3β inhibition, which (in concert with AMPK), phosphorylates and directly activates TSC leading to stimulation of mTORC1 activity [63]. The Wnt coreceptor LRP6 can also increase aerobic glycolysis in a β-catenin-independent manner by directly activating AKT-mTORC1 signalling. Additionally, Wnt5a also increases aerobic glycolysis, mediated by the AKT-mTORC1 signalling module and regulates mitochondrial fission-fusion processes [63].

2.7. Molecular background of LAM (mTORC1/2 formation and downstream signalling)

When mTOR is activated *two complexes* can form: mTORC1 or/and mTORC2. The composition of mTORC1 is the complex of mTOR (catalytic subunit of the complex); regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein (mLST8); proline-rich AKT substrate (PRAS) 40 kDa; and DEP-domain-containing mTOR-interacting protein (Deptor) (Figure 3). The roles and functions of most of the subunits in mTORC1 formation remain unclear. The role of Raptor has been explained as a regulator for the assembly of the substrate for mTOR protein [64]. Both PRAS40 and Deptor are negative regulators of mTORC1. When the amount of mTORC1 decreases both PRAS40 and Deptor starts to bind to the complex. The function of mLST8 is still

unclear. Recent data have shown that knocking out mLST8 does not influence normal mTORC1 formation and does not affect mTORC1 function. mTORC1 phosphorylates PRAS40 and Deptor destabilize their interaction with mTORC1 and modify the downstream signals of the mTOR pathway [65].

The structure of mTORC2 complex is highly similar to mTORC1. mTORC2 is formed of six different proteins most of them shared with mTORC1 including mTOR, Deptor and mLST8. Unique proteins for mTORC2 include mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1) [64]. Rictor and mSIN1 are responsible for forming the main structure of mTORC2, while they also stabilize each other. Just like in mTORC1, Deptor acts as an endogenous inhibitor in the mTORC2 complex. Finally, mLST8 is required for the stability of mTORC2 function, deleting mLST8 resulting in deregulation and destabilization of mTORC2 [55]. Interestingly, within the mTORC2 complex, mTOR also functions as a tyrosine kinase that phosphorylates and activates insulin receptors and insulin-like growth factor 1 (IGF1) receptors [55]. mTORC2 has also been implicated in the control and maintenance of the actin cytoskeleton [66]. Rapamycin inhibits mTORC1 activity, which is up-regulated in LAM cells due to mutations in TSC1 and/or TSC2 genes that are both regulators of mTORC1.

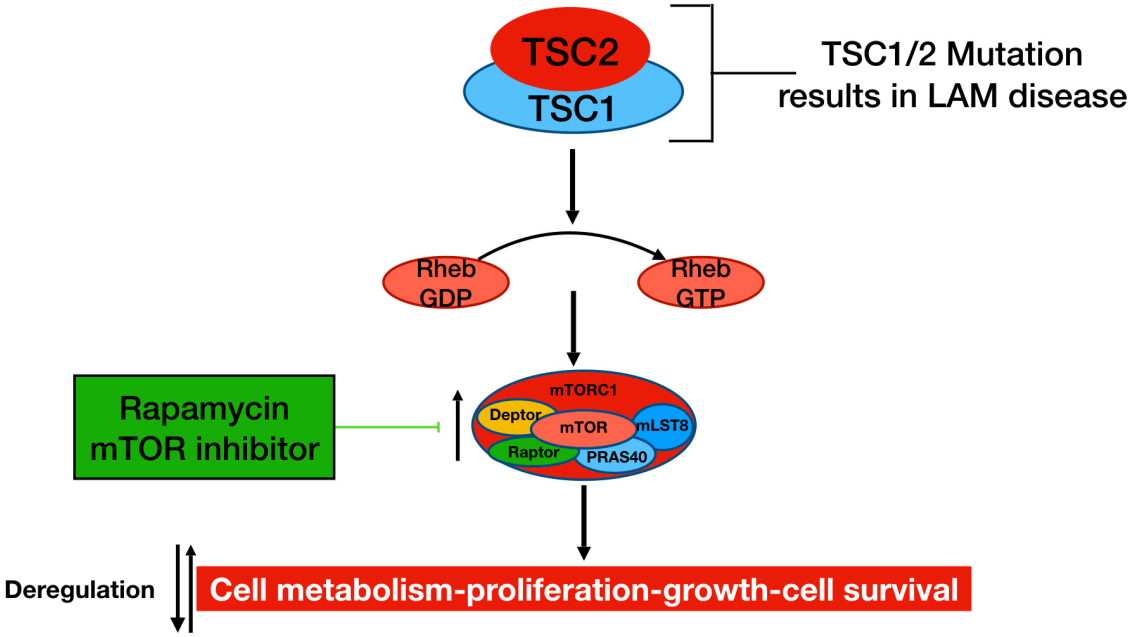


Figure 3. mTORC1 signalling pathway. TSC1/TSC2 complex is a key regulator of mTORC1. It acts as a GTPase activating protein for Rheb, turning off mTORC1 activation by Rheb [65].

mTORC1 positively regulates cell growth and proliferation, and protein synthesis by increasing anabolic processes and inhibiting catabolic processes. Anabolic processes include biosynthesis of proteins, lipids and organelles, while catabolic processes include autophagy. mTORC1 assists protein synthesis via phosphorylation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and the ribosomal protein S6 kinase β-1 (S6K1) [23]. The phosphorylation of 4E-BP1 inhibits its binding to eIF4E allowing eIF4E to assist initiation translation mechanism (cap-dependent). Increase of S6K1 expression depends on the activity of mTORC1 which leads to an increase in mRNA biogenesis and translation of ribosomal proteins via regulation of the activity of three genes: nuclear chaperone protein S6K1 aly/REF-like (SKAR) target of S6K, programmed cell death 4 (PDCD4) and eukaryotic elongation factor 2 kinase (eEF2K) [67]. Ribosome biogenesis can also be stimulated by mTORC1 via activation of protein phosphatase 2A catalytic subunit alpha (PP2A) and the transcription factor IA (TIF-IA) [67]. These proteins are necessary for cell growth and proliferation, therefore deregulation of mTORC1 can stimulates uncontrolled cellular proliferation in cancers. mTORC1 also contributes to lipid synthesis via increasing the activity of sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor-γ (PPARγ) [68]. Two factors that control the gene expression level of proteins involved in lipid and cholesterol homeostasis. Knocking down mTOR reduces the expression and transactivation activity of PPARγ, PPARα and PGC1-α [69] [68] leading to deregulation of lipid homeostasis. Another downstream effect of mTORC1 activity is ***autophagy*** [33]. Autophagy is a breakdown process that is important in degradation of dysfunctional cellular organelles and improvement of protein turnover and is carried out by autophagosomes and lysosomes [70]. When a cell is under stress or shortage of nutrients, degradation of organelles and protein complexes take place to provide enough biological material to assist anabolic processes such as protein synthesis and energy production [71]. mTORC1 enhances anabolic activity in a cell via antagonizing autophagy. The cross talk between autophagy and mTORC1 is not entirely clear yet. It is known, however, that inhibition of mTORC1 can enhance autophagy and *vice versa* [72]. Earlier studies have implicated for example unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) in the crosstalk between autophagy and mTORC1 [72], but a detailed pathway analysis is still awaits further investigation. ***Mitochondrial metabolism and biogenesis*** are both regulated by mTORC1. A strong connection between mTORC1 and mitochondrial activity has been found in earlier studies [73]. A significant increase in mitochondrial

DNA copy number and higher expression of many genes encoding proteins involved in oxidative metabolism were recorded after constant activation of mTORC1. In contrast, inhibition of mTORC1 by Rapamycin resulted in significant decrease in mitochondrial DNA copy number [74][75]. Based on the above studies it was suggested that mTORC1 activity regulates expression of PGC-1α and ERRα [75]. Similarly, transcription factor yin-yang 1 (YY1), that is crucial in mitochondrial biogenesis and oxidative metabolism, is also a common target of mTOR and PGC-1α [75]. Another study proved a correlation between cell proliferation and energy demands via complex formation amongst mTORC1-Eif4e binding protein (d4E-BP) and mRNAs encoding genes of mitochondrial respiration. The mitochondrial genes included complex V, cytochrome C (CYP) and mitochondrial transcription factor A (TFAM) [73]. Decreased respiration and higher glycolysis levels (Warburg effect) in TSC deficient cells has also been demonstrated upon mTORC1 activation (Figure 4) [76]. It was shown that mTOR regulates mitochondrial activity independently of any other cellular targets [76] and mTOR activity can play an important role in determining the relative balance between mitochondrial and non-mitochondrial sources of ATP generation. mTORC1 affects the metabolism by a shift in glucose metabolism from oxidative phosphorylation to glycolysis. mTORC1 controls this shift via the activation of the transcription factor HIF1α which drives the expression of several glycolytic enzymes such as phospho-fructo kinase (PFK) [77].

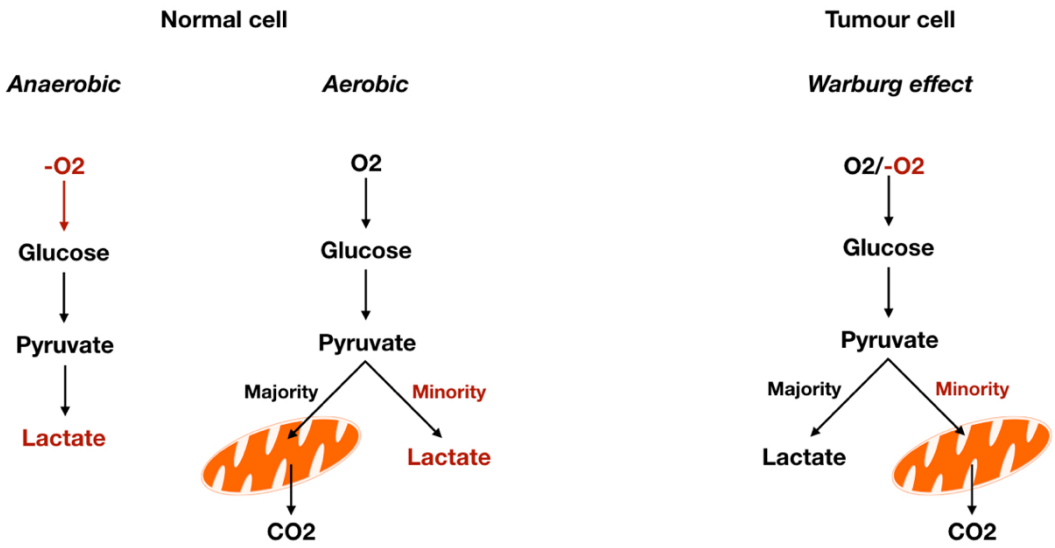


Figure 4. Schematic illustration of cellular respiration in normal and cancer cells.

mTORC2 has been characterized as a Rapamycin-insensitive target. Rapamycin-insensitivity and low survival rate of the mTORC2 knockout mouse model still leaves the role of mTORC2 signalling largely unexplained. Yet, many studies point out the importance of mTORC2 in biological processes including cellular survival, metabolism and proliferation [78]. Two factors that are known to trigger mTORC2 signalling are GF and insulin. Upon binding to their receptors they activate IRS1-PI3K-mSIN1-mTORC2. Signals downstream of mTORC2 target and activate AKT and SGK1. AKT phosphorylates TSC2 (also affecting mTORC1) and GSK3β (an important component of the Wnt signalling pathway) [65]. SGK1 inhibits FoXO1 and FoxO3a, two genes that are known for their role in stress resistance, metabolism, cell-cycle arrest and apoptosis [79]. mTORC2 also regulates cytoskeletal reorganization by affecting actin via protein kinase C-alpha (PKCα) and paxillin phosphorylation [73]. mTORC2 participates in mitochondrial activity via activation of AKT.

Upon AKT activation mTORC2 is relocated to a sub-compartment of the endoplasmic reticulum (ER) termed mitochondria-associated ER membrane (MAM), where mTORC2 can interact with the IP_3 receptor (IP3R)-Grp75–voltage-dependent anion-selective channel 1 ER-mitochondrial tethering complex. Deficiency in mTORC2 activity results in breakdown of mTORC2-MAM formation leading to suppression of mitochondrial ATP production, membrane potential, and Ca^{2+} uptake [80].

2.8. Cellular Metabolism - the mitochondria

Most studies of various pathologies have reported changes in the metabolic activity of diseased cells. In the centre of the metabolic malfunction is the dysfunctional mitochondria. The malfunctioning, often hyperactive mTOR has been identified to drive mitochondrial respiration, glycolysis [24] and uncontrollable proliferation [81]. It is well known that mitochondria are cell organelles bound by a double membrane. In metabolically active cells mitochondria occupy a considerable percentage of the cell volume and its main function is to produce ATP. Mitochondria produce ATP by oxidizing products of glucose, pyruvate and the reduced form of nicotinamide adenine dinucleotide (NADH) in the presence of oxygen. This procedure is known as aerobic respiration. In the case of oxygen depletion mitochondria uses anaerobic fermentation of glucose resulting in a lower yield of ATP [82]. The double mitochondrial membranes contain the outer and inner membrane, intermembrane space, cristae and the matrix. The *outer membrane* is a thick enclosure that keeps the structure and the integrity of the

mitochondria. It also contains porins that control the traffic of molecules into the inner structure. Intermembrane space, also known as pre-mitochondrial space, controls transport of proteins, electrons and metal ions. It also contains cytochrome C, a haeme protein that transfers electrons between respiratory chain complexes. The *inner membrane* performs the major functions including oxidative phosphorylation, generation of ATP in the matrix, regulation of metabolite and protein movement to the matrix and controls fusion of mitochondria [83]. The cristae is not a separate compartment of the inner membrane, it just covers the inner face of the inner membrane enhancing its ability to produce ATP. The mitochondrial matrix is surrounded by the inner membrane and contains nearly 60% of the mitochondrial proteins, enzymes, mitochondrial ribosomes and tRNA that are needed in ATP production (Figure 5).

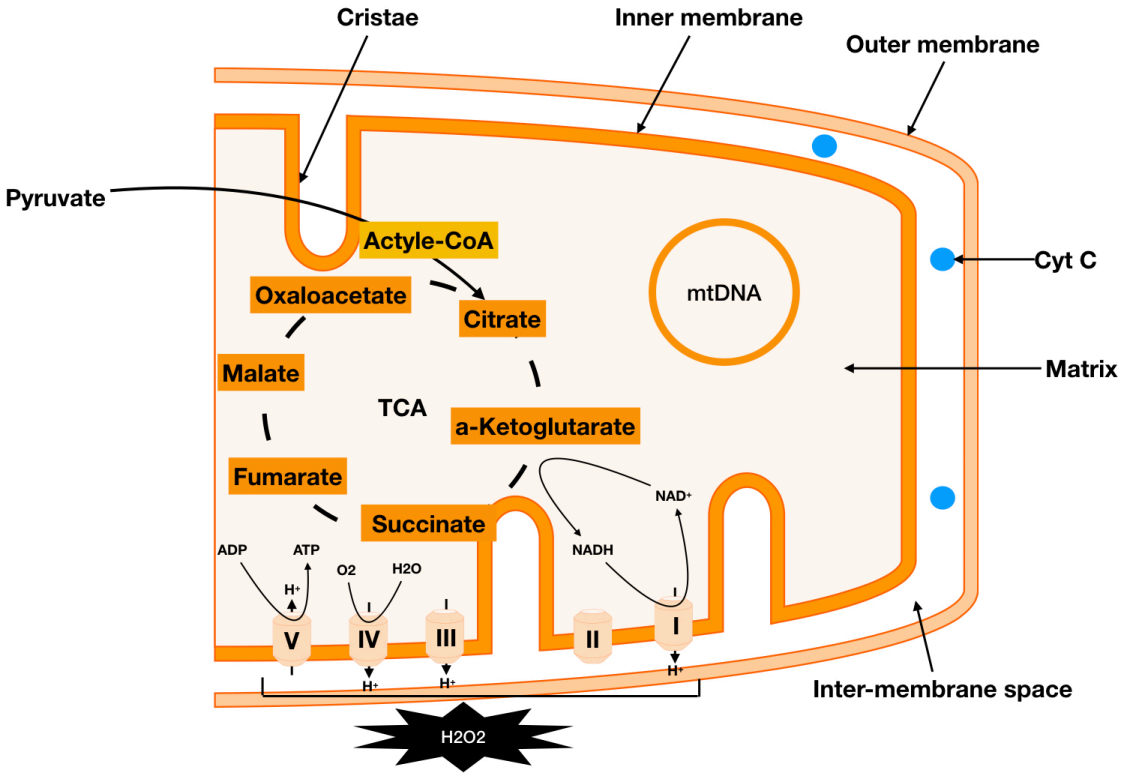


Figure 5. Schematic representation of mitochondrial structure, TCA cycle and the electron transport chain (ETC) [83] [82].

The mitochondrial genome is 16569 bp, and encodes 37 genes important for the respiratory chain and ATP production. These genes code components of complexes I-III-IV-V of the ETC, as well as tRNA and genes for mitochondrial replication and repair [84]. Apart from mitochondrial genes mitochondrial function and biogenesis also fall under the regulation of nuclear genes. Nuclear genes that control mitochondrial function include mitochondrial transcription factor A (TFAM) and nuclear respiratory factor 1

(NRF1) [85]. TFAM encodes a key mitochondrial transcription factor containing two high mobility group motifs. The encoded protein also functions in mitochondrial DNA replication and repair. NRF1 is another transcription factor with the ability to activate metabolic genes required for mitochondrial respiration, DNA transcription and activation of TFAM [85].

In a healthy cell, all events such as proliferation, migration, cell division, regulation of cell volume, maintenance of cell architecture and autophagy require an adequate amount of energy. The relation between cellular events and metabolism (including energy production and respiration) falls under strict regulation. In cancer cells the cellular events demand higher amount of ATP for fuelling the increased proliferation rate. The majority of ATP in cancer cells is produced by oxidative phosphorylation (OxPhos) [86]. OxPhos is a metabolic pathway where cells use enzymes to oxidize nutrients to yield high levels of ATP. However, cancer cells in many cases produce ATP via anaerobic fermentation with higher glycolysis levels even in the presence of oxygen (Warburg effect) [87][88]. Deregulation of the metabolic activity of mitochondria can be the result of mutation in the mitochondrial DNA, and/or mutation or malfunction of the nuclear genes regulating mitochondrial function [89].

In cancer, metabolic dysfunction, especially mitochondrial dysfunction, is part of the disease process [89]. There have been several attempts to target the mitochondria in cancer therapy using metabolic inhibitors [90]. 3-bromopyruvate (3BP) is an example of strong glycolysis inhibitor that binds the hexokinases localized on the mitochondrial membrane with very high selectivity [91]. 3BP inhibits mitochondrial glycolysis and monocarboxylate transporter 1 (MCT1) that regulates lactate export of cancer cells [87]. New evidence has also pointed to a connection between resistance to chemotherapy and deregulation of metabolic activity [93]. In breast, cervical and colon cancers both lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 3 (PDK3) contribute to paclitaxel and trastuzumab resistance [94]. The resistance to the oldest of chemotherapeutic drugs cisplatin, has also been associated with mTOR associated pathways in gastric cancer [95]. Additionally, the metastatic processes of cancers are linked to metabolic changes and increased aerobic glycolysis [88]. The increased aerobic glycolysis leads to the overload of the electron transport chain and superoxide production [87]. If superoxide scavengers could be used in therapy, then they could decrease the overload of the electron transport chain, block tumour migration and stop tumour metastasis [96].

2.9. Current Therapies for LAM

For a long time, LAM has been considered as a fatal disease with only a limited options for clinical intervention. Even currently, the management of LAM primarily focuses on controlling the clinical symptoms. As LAM progresses pneumothoraces develop and the abnormal collection of air in the pleural space is removed using chest tube drainage, pleurodesis and pleurectomy with the aid of video-assisted thoracoscopy [97]. Chylous effusions (composed of chyle, the lymphatic fluid that flows through the thoracic duct in the chest) and lymphangioleiomyomas can also be detected in LAM patients that may be treated with continuous tube drainage of the chylous pleural effusions, thoracostomy and surgical pleurodesis [4]. Finally, when lung function declines drastically, lung transplantation becomes the last resort for therapy [98]. Lung transplantation, however, is a high risk procedure and recurrence of the disease after transplantation is frequent as the origin of the disease is unknown and long lasting targeted therapy is not available [98].

Detailed molecular research has, however, led to the first targeted treatment of LAM. The first drug to be prescribed to LAM sufferers is the mTOR inhibitor, Rapamycin (Sirolimus) [4] that stabilizes lung function in most patients for a considerable length of time, but it does not offer progression-free survival or a cure [99]. mTOR functions as a serine/threonine kinase regulating [73] cell proliferation, motility, survival, transcription, protein synthesis and autophagy [77]. mTOR deregulation has been linked to increased angiogenesis, insulin resistance, adipogenesis, T-lymphocyte activation [68] as well as carcinogenesis, type 2 diabetes and cyst formation [64]. TSC1 and/or TSC2 mutations occur in both forms of LAM disease: inherited-LAM (TSC-LAM) or sporadic form LAM (S-LAM). Mutations in both TSC genes lead to up-regulation of mechanistic target of rapamycin complex 1 (mTORC1) [11]. Perhaps therefore it is not surprising that mTORC1 inhibitors offer effective stability for lung function, reduce the bulk of lymphangioleiomyomas, chylous effusions and AMLs, and increase autophagy [5]. As many signalling pathways interact with mTOR identification of additional therapeutic targets remains a prime focus of research [65].

A major drawback of Rapamycin treatment is adverse effect. In more than 30% of patients peripheral edema, hypercholesteremia, hypertension, hypertriglyceridemia, increased creatinine and thrombocytopenia leading to a 5% treatment discontinuation. To make LAM therapy even more difficult, discontinuation of Rapamycin increases the symptoms rapidly and the cysts grow back to their original size. Another drawback of rapamycin is that it has no effect on mechanistic target of rapamycin complex 2 (mTORC2) [78]. Some clinical studies have claimed that control of serum estrogen levels offers up an alternative route to prevent disease progression, although such attempts have mostly failed [100]. Many studies pointed out different therapeutic targets e.g. vascular endothelial growth factor receptor 3 (VEGFR3), VEGFD, mitogen-activated protein kinase (MEK) and MMP [101][20] but no effective therapy has been developed to target any of the above molecules.

3. AIMS

In the present research it was the primary aim to understand the molecular background of LAM in more detail which understanding can lead to potential identification of novel therapeutic targets. The aims can be grouped into three main areas:

1- To investigate the underlying hormone dependency of LAM disease.
2- To investigate the role of Wnt signalling in the modulation of LAM disease development and progression.
3- To test novel drugs that have shown characteristic efficacy in other characteristically female diseases (e.g. breast cancer).

4. MATERIALS AND METHODS

4.1. Ethical Statement

Lung tissue samples were obtained from human lung transplant donors, in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the University of Pennsylvania [102]. Human tissue was obtained from National Disease Research Interchanged (NDRI, Philadelphia, PA, USA). LAM patients had given written consent and all the collected samples were treated anonymously.

4.2. LAM cell lines, bronchial Smooth Muscle Cells (SMC) and cell culture conditions

Primary cultures of human LAM cells where established in the Department of Medicine, University of Pennsylvania, Pennsylvania, USA [102]. Briefly, the primary cultures of LAM cells were dissociated from the LAM nodules of transplant patients. Each LAM nodule was used to establish individual cell lines (characterized based on alpha smooth muscle actin (α-SMA) expression, mTORC1 activation, HMB45 immunoreactivity, DNA synthesis, TSC2-mTOR-PS6-MLANA genes, and cell migration) [103]. In the current study LAM cell lines were derived from individual patients and identified as LAM-100, LAM-111C, LAM-D9065 and LAM-HUP. As controls, primary cultures of normal, human bronchial smooth muscle cells (SMC), were purchased from Lonza (Basel, Switzerland). Normal, bronchial SMC and LAM cells were cultured at 37°C, 5% CO_2 in SMC Growth Medium (insulin, hFGF, GA, FBS and hEGF) (Lonza, Basel, Switzerland). Primary, normal, bronchial SMC cells from two individual donors and the four individual cell lines were characterized using ***α-***SMA staining and testing various (PS6, MLANA, mTOR) gene expressions.

4.3. Hematoxylin eosin staining

Cytospins of normal, bronchial SMC and LAM cell lines were stained in Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, USA) for 10 min, washed, then differentiated with 0.25% acetic acid and in eosin solution. Sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, USA). Images were taken using Nikon Eclipse Ti-U inverted microscope.

4.4. Electron microscopy

Cells were resuspended in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) for 24h, rinsed in 0.1 M sodium-cacodylate buffer and pelleted. The pellet was

embedded in Spurr low-viscosity resin with ERL 4221 used as the epoxy monomer and cured at 70ºC for 16h. For transmission electron microscopy (TEM), 90 nm thick sections were stained with alcoholic uranyl-acetate and Reynolds lead-citrate and examined using Jeol 1200 and Jeol 1400 transmission electron microscopes (Jeol Ltd, Tokyo, Japan) at 80 kV. Images were acquired using an integrated MegaView III digital camera (Olympus Soft Imaging Solutions GmbH; Munster, Germany).

4.5. Treatments

4.5.1 Proxison treatment

Normal, bronchial SMC and LAM cell cultures were treated with 3 μM Proxison (Antoxis Ltd, Aberdeen, UK) for 1h at 37°C, 5% CO_2 [96]. In Proxison and Rapamycin combination treatment (migration assay, mitochondrial genes qRT-PCR), cells were treated with 3 μM Proxison and 20 nM Rapamycin for 24 h at 37°C, 5% CO_2.

4.5.2. Retinoic acid treatment

Normal, bronchial SMC and LAM cell cultures were treated with 1 and 2 μM retinoic acid (Sigma-Aldrich, St. Louis, USA) for 24h at 37°C, 5% CO_2 [104]. In retinoic acid and Rapamycin combination treatment (scratch assay), cells were treated with 2 μM retinoic acid and 20 nM Rapamycin for 24 h at 37°C, 5% CO_2.

4.5.3. Lutein treatment

Normal, bronchial SMC and LAM cell cultures were treated with 100 μM Lutein (Department of pharmacognosy, university of Pecs, Hungary) for 24h at 37°C, 5% CO_2 [105].

4.5.4. Rapamycin treatment

Normal, bronchial SMCs and LAM cell cultures were treated with 20 nM Rapamycin catalog: tlrl-rap (InvivoGen, San Diego, USA) for 24h at 37°C, 5% CO_2 in monotherapy [106]. Rapamycin pre-treatment was made for 48h (20nM/24h) then 3 μM Proxison was added for an extra 24h.

4.6. Flow cytometry

Normal, bronchial SMC and LAM cells (100,000) were collected from Proxison treated and control cultures. Cell cultures were incubated with 2.5 μM of Rhodamine 123 (RH-123) (Sigma) for 30 min at 37ºC, then cooled to 4ºC and washed twice with PBS. Viability

was tested using propidium iodide (PI) staining (Invitrogen, Ltd). Cells were analysed using FACS Canto II flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) with BD FACS DIVA software V6 and data were analyzed by FCS Express V3 software.

4.7. RH-123 fluorescence microscopy

Normal, bronchial SMC and LAM cells were cultured for 3 days using Falcon™ chambered cell culture slides (Thermo Fisher Scientific, Waltham, USA), then treated with Proxison as described above. Images from living cells were acquired using an Olympus IX-81 (OLYMPUS Corp., Tokyo, Japan) light and fluorescent microscope, then densitometry was performed using ImageJ.

4.8. Immunofluorescent staining

Normal, bronchial SMC and LAM cells were cultured for 3 days using Falcon™ chambered cell culture slides (Thermo Fisher Scientific, Waltham, USA). Cell cultures were then fixed with 4% formaldehyde and permeabilized with PBS containing 0.1% Triton-X and 5% BSA.

Anti-alpha -Smooth Muscle Actin	MAB1420 (1:100)
Anti-mTOR Antibody	ab25880 (1:100)
Anti-p70 S6 kinase Antibody	SC-8416 (1:100)
Anti-mouse Alexa 488	A28175 (1:200)
Anti-rabbit Alexa 488	A11034 (1:200)
Anti-mouse Alexa 647	A32728 (1:200)

Nuclei were counter stained with DAPI. Images were acquired using an Olympus IX-81 (OLYMPUS Corporation, Tokyo, Japan) both light and fluorescence microscope.

4.9. Migration assay

200,000 cells of both normal, bronchial SMC and LAM cell lines were seeded in serum-free media using various treatments in the upper chamber of the Transwell migration plate. The pore size of the membrane was 8.00 μm (24-well format, Costar, Corning Incorporated). The chambers were incubated at 37^{o}C for 16 h. Chambers were fixed in PBS containing 4% paraformaldehyde, stained with DAPI and membranes were mounted to microscopic slides (supplemental figure 6). Images were acquired using an Olympus IX-81 (OLYMPUS Corporation, Tokyo, Japan). The number of migrated cells was analysed using ImageJ particular analyser.

4.10. RNA isolation

Total RNA was extracted from normal, bronchial SMC and LAM cell cultures with MN NucleoSpin RNA isolation kit according to the manufacturer's protocol (Macherey-Nagel, Düren, Germany). The concentration of RNA samples was measured using NanoDrop (Thermo Fisher Scientific, Waltham, USA).

Total RNA from human lung tissues were obtained using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, USA). RNA (1 μg) was digested with DNase (Sigma-Aldrich, St. Louis, USA) to eliminate any DNA contamination.

4.11. TaqManArray, Nanostring and Quantstudio chips

4.11.1. Human Nuclear Receptors TaqMan®Array

cDNA was synthesized with high capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, USA). Reverse transcription was performed with random hexamer primers. Each sample was mixed with TaqMan Universal Master Mix (Thermo Fisher Scientific, Waltham, USA). TaqMan PCR reaction was performed using ABI StepOnePlus system and data were analyzed with StepOne software. MicroRNA expression was normalized to U6 expression.

4.11.2. Nanostring

100 ng of total RNA/cell culture was isolated and analysed using the nCounter Analysis System (NanoString Technologies) and the nCounter Human v2 miRNA Panel containing 798 unique miRNA barcodes. Copy count assay was performed using the Nanostring ncounter SPRINT. Analysis was performed using the Nsolver software.

4.11.3. Quantstudio 12k flex

cDNA was prepared using TaqMan miRNA reverse transcriptase kit and Megaplex RT primers Pool A and B (Thermo Fisher Scientific, Waltham, USA) according to manufacturers' protocol using 350ng-1000ng of total RNA as starting material. miRNA expression levels were assessed using open array miRNA card Pool A and B and Quantstudio 12k flex (Thermo Fisher Scientific, Waltham, USA).

4.12. Protein array

4.12.1. Angiogenesis array

Cell lysates of 1 x 10^7 cells/ml were assessed using a Human Angiogenesis Array Kit (R&D Systems, Minneapolis, USA). Protein concentration was determined using a fluorescent protein assay (Qubit Protein, Thermo Fisher Scientific, Waltham, USA). Briefly, the Detection Antibody Cocktail was mixed with each sample and incubated with the membrane at 4°C overnight, then with Streptavidin-HRP at room temperature finally with the Chemiluminescent-Reagent Mix. Images were captured using LAS-4000 (GE Healthcare Bio-Sciences AB Uppsala, Sweden), and intensity was determined using ImageJ (https://imagej.nih.gov/ij/) and normalized to the reference spots.

4.13. Quantitative qRT-PCR

cDNA was synthesized as described above. qRT-PCR was performed using SensiFAST SYBR Green reagent (BioLine, London, UK) in an ABI StepOnePlus system. Gene expressions using sequence specific primers (Table 1) were analysed with StepOne software and normalized to beta-actin. Changes in gene expression were calculated according to the 2^{-ddCt} method.

Gene name	Forward primer	Reverse primer
β-actin	GCGCGGCTACAGCTTCA	CTTAATGTCACGCACGATTTCC
NRF1	CAGCCGCTCTGAGAACTTCA	TTCCCGCCCATGCTGTTTAT
Cyt C	TCAGGCCCCTGGATACTCTT	AAGTCTGCCCTTTCTTCCTTC
COX4	GTTTCACCGCGCTCGTTATC	TTGGCCACCCACTCTTTGTC
VEGFD	GAACACCAGCACCTCGTACA	ACAGACACACTCGCAACGAT
VEGFC	CCCGCCTCTCCAAAAAGCTA	TGGACACAGACCGTAACTGC
VEGFA	TTCTGGGCTGTTCTCGCTTC	TTGTCACATACGCTCCAGGAC
VEGFR1	ACCATACCTCCTGCGAAACC	TCAGAGGCCCTTTCAGCATT
VEGFR2	CGGTCAACAAAGTCGGGAGA	CAGTGCACCACAAAGACACG
VEGFR3	TGTACACCACGCAGAGTGAC	AGCCTTTGTAGGTCGTTGGG
TFAM	CTTATAGGGCGGAGTGGCAG	CAGCTTTTCCTGCGGTGAAT
TSC1	CCGTGGCCCTATGCTTGTAA	CGGCTTTGCCCACATATTCG
TSC2	CCTTGGACGGTATTGCCTGT	GCCTGCTTCTGTGTACCACT
HIF1-α	GTCTGAGGGGACAGGAGGAT	GCACCAAGCAGGTCATAGGT
ESR1	GACTGCACTTGCTCCCGT	CCACTTCGTAGCATTTGCGG

THRB	AGGGCACTGGTAATTTGGCT	TGGCTTTGTCACCACACACT
NR0B1	GACTGTGGAAGTCTCGGAGC	ACTTGATGGCTTGGACCTGG
NR5A2	CCCAAGGCCACGAAATTTGA	GCCCAGCACCAATAGGTGTAA
ESRR*γ*	AATAATGGTTGCCGGTCGCA	TGCAGAGAAGCTCTTCCTCGTAG
AHR	CCACTTCAGCCACCATCCAT	AAGCAGGCGTGCATTAGACT
MLANA	CTGCTCATCGGCTGTTGGTA	GAGACACTTTGCTGTCCCGA
MTOR	AACCTCCTCCCCTCCAATGA	TCAGCGGTAAAAGTGTCCCC
RPS6	TGTTACTCCACGTGTCCTGC	AAGTCTGCGTCTCTTCGCAA
RARβ	ATCCGAGCAGGGTTTGTCTG	TTTTCCCAGCCCCGAATCAT
PGR	TGCCTGAAGTTTCGGCCATA	AAGCGGGAATCTTCCTTGGG
ALDH1A1	GATCCCCGTGGCGTACTATG	TGGATCTTGTCAGCCCAACC
RDH	GGAATCAGCCCCATCAAGGA	AGGCAAGGCTGGAAGGTTTT
BCO1	AGATGGCCAAGTCTACTGCC	ATCCTCATCCTTGGCACCTG
BCO2	CAGTTCTGTGCGTGTTCACTG	GCAGTTGCTCCATTCACAGC

4.14. *Metabolic profiling*

4.14.1. Metabolic profiling using SeaHorse XF96

Normal, bronchial SMC and LAM metabolic profiles were generated using the Seahorse X96 platform (Agilent Technologies, USA) [107]. Briefly, cells were plated into Seahorse cell plates, then at 90% confluence and after recording baseline oxygen consumption, cells were treated with butyril-cAMP (500 μM), oligomycin (2 μM) and antimycin (10 μM). Antimycin-resistant oxygen consumption was considered as baseline oxygen consumption, and membrane leak (OCR after oligomycin treatment) was calculated. Glycolysis was assessed through the extracellular acidification value (ECAR) and ECAR/OCR values were calculated.

4.14.2. Metabolic profiling using Oroboros

LAM and normal, bronchial SMCs respiration was measured using a high resolution Oxygraph-2k (O2k, OROBOROS Instruments, Innsbruck, Austria). Oxygraph-2k chambers were filled with respiration medium and the chamber was allowed to equilibrate and the baseline built. Cells (10,000,000) in smooth muscle growth medium from both SMC and LAM were injected into the chamber. To generate a closed system Antimycin was inserted to stop cellular oxygen consumption [108].

4.15. TRXR activity

Cell lysates (1 x 10^7 cells/Ml) were collected to assess TrxR activity using a Thioredoxin Reductase Assay Kit (Abcam, Cambridge, MA, USA). Protein concentration was determined (Qubit Protein, Thermo Fisher Scientific, Waltham, USA), then a TrxR activity assay was performed according to the manufacturer's instructions. OD was measured at 412 nm.

4.16. Artificial neural network (ANN) analysis

Gene expression data of nuclear receptors and the angiogenesis protein array were evaluated using a feed forward artificial neural network (ANN) (Neurosolutions 6, NeuroDimension Inc.) software [109][110].

4.17. Statistical analysis

Statistical analysis was performed with SPSS version 20 software. Data are presented as mean ± standard error of mean (SEM), and statistical analysis was performed using the independent samples t-test and one-way ANOVA with Bonferroni correction. $p<0.05$ was considered as significant.

5. RESULTS AND DISCUSSION

5.1. Morphological study and characterisation of LAM cell lines

LAM cell lines (LAM100, 111c, D9065, HUP) used in the study were isolated from four LAM patients undergoing lung transplantation in Philadelphia, USA (National Disease Research Interchange (NDRI), Philadelphia, PA, USA). LAM cell lines carrying TSC2 mutations demonstrated hyperactive mTORC1 signalling in approximately 76% of cells and demonstrated high proliferation rate, increased migration and invasiveness, characteristic to neoplastic cell lines, compared to the same passage number of primary human lung fibroblasts during cell characterisation (Dr Krymskaya (Perelman School of Medicine, University of Pennsylvania, USA). Two normal SMC cell lines were isolated from healthy lung donors and were purchased from Lonza. SMCs were tested for *a*-SMA and von Willebrand Factor (Factor VIII) by the manufacturer. To compare the normal and diseased LAM cell lines, haematoxylin eosin and *a*-SMA IF staining was performed (Figure 6). Using the above techniques, no significant differences were detected between LAM cells and SMCs (Figure 6A, B).

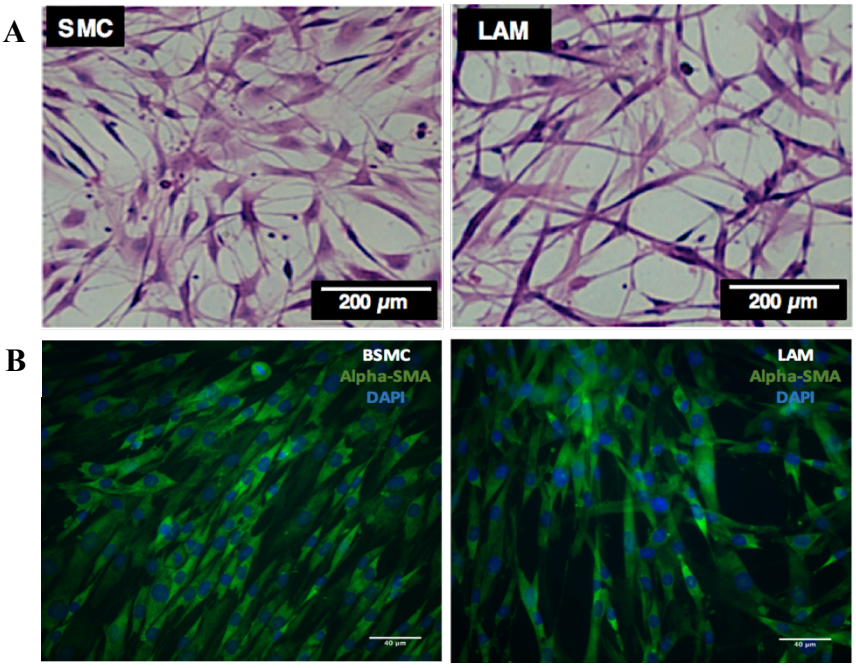
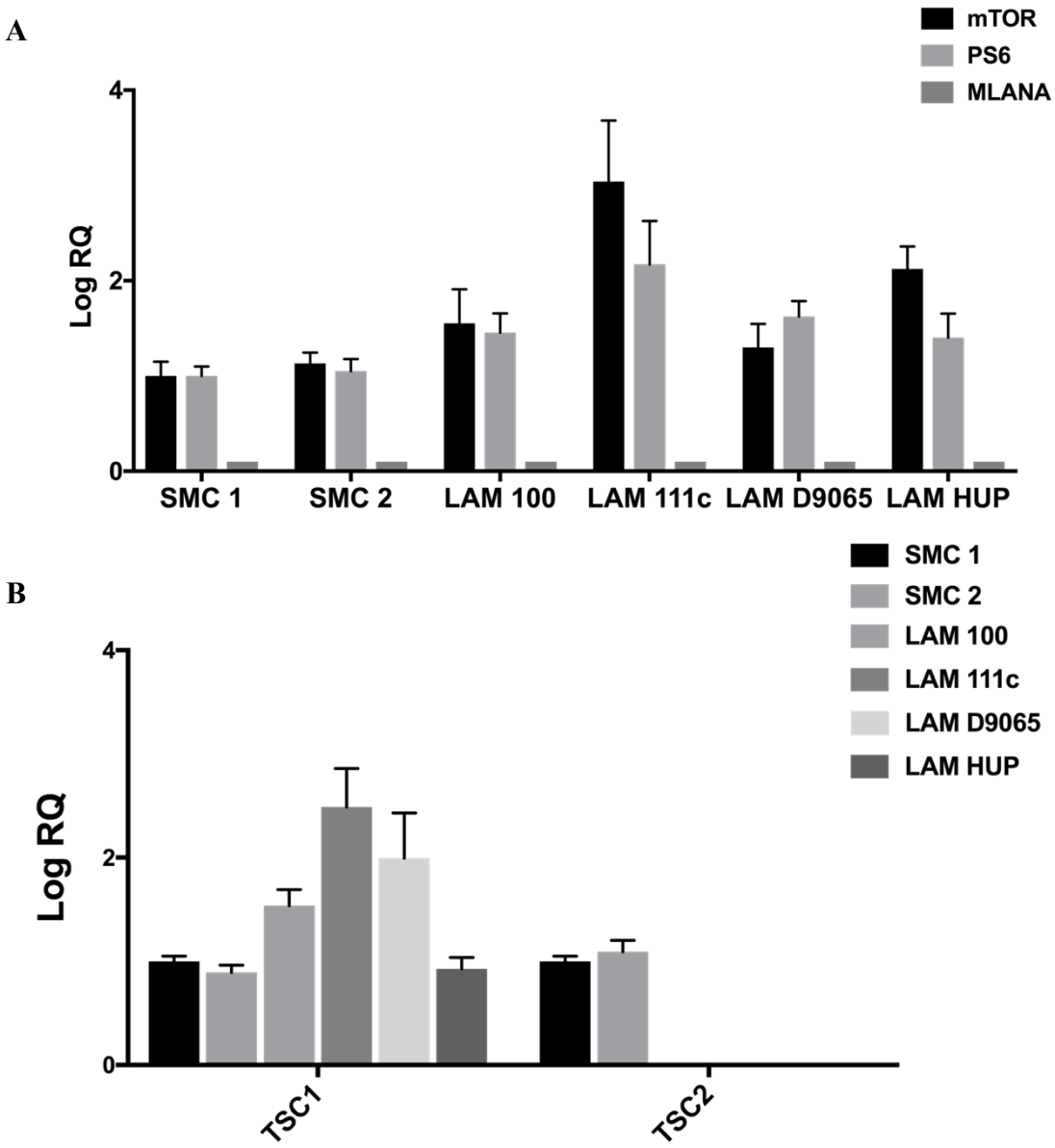


Figure 6. Morphological characterization of BSMC (n=2) and LAM (n=4) cell lines. **A)** Haematoxylin eosin (magnification 10x, size-bar 200 µm). **B)** ***a*-**SMA (***a*-SMA green, DAPI blue, magnification 20x, size-bar 40 µm).

To characterise the molecular differences, qRT-PCRs of HMB-45, mTOR, TSC2 and PS6 genes were performed and confirmed with immunofluorescent staining. Significant up regulation of mTOR (originally reported by Dr Krymskaya et al) [102] and PS6 genes in LAM cell lines were detected compared to normal SMCs controls (Figure 7A, C, D). Also, a total loss of TSC2 gene expression was observed in LAM cells (Figure 7B). As both TSC1/2 genes are essential for regulation of the mTOR pathway, the observation that the TSC2 gene produced no transcript was an important factor to use these patient derived cell lines as models of LAM disease.



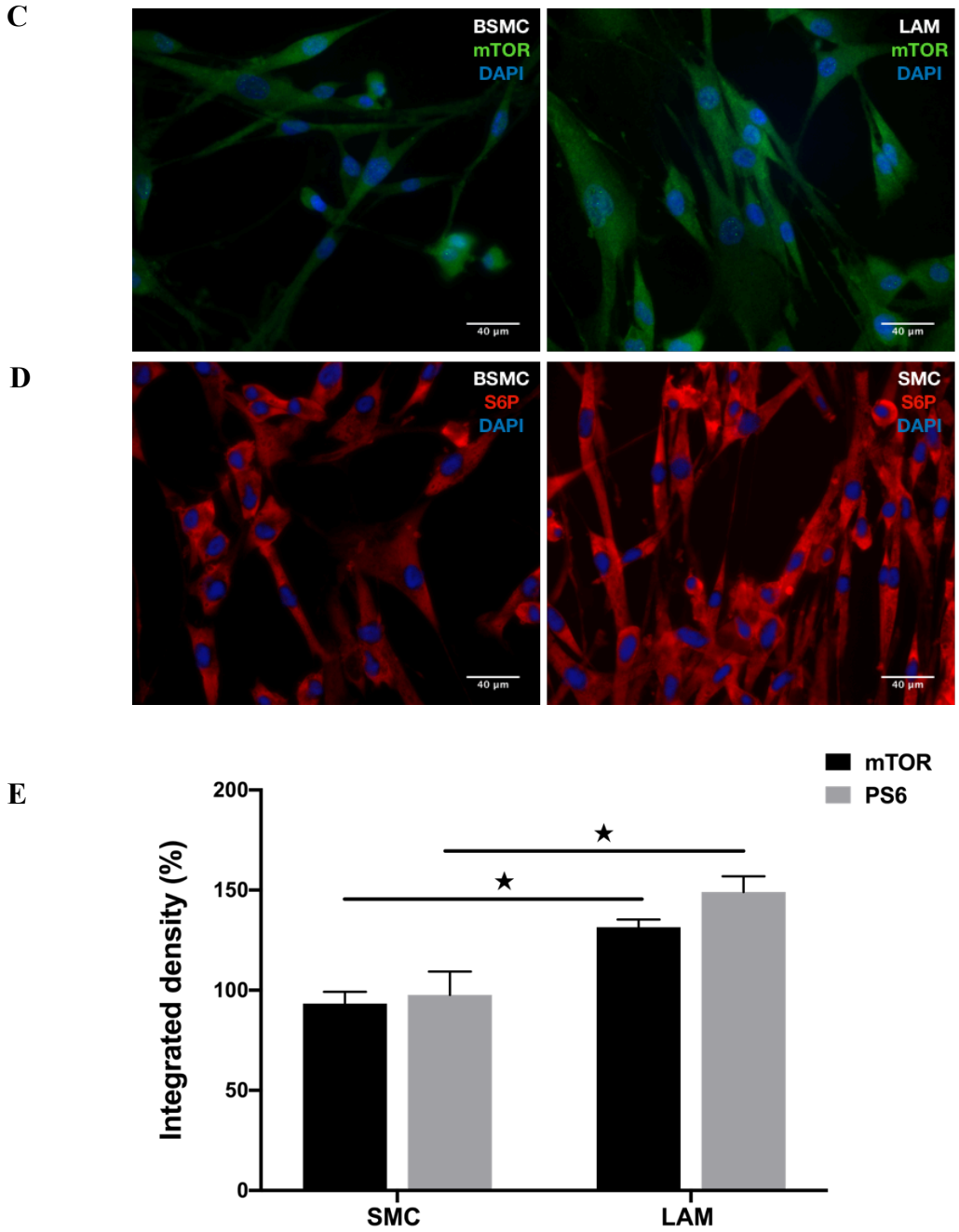


Figure 7. Molecular Characterization of normal bronchial SMC cells (n=2) and individual patient derived LAM cell lines (n=4). **A)** qRT-PCR analysis of mTOR, MLANA and PS6 genes. Beta-actin was used as inner control. Data are presented as mean of log RQ ± technical error of the replicates. **B)** qRT-PCR analysis of TSC 1 and 2 genes. Beta-actin was used as inner control, loss of TSC2 gene expression was significant in LAM patient cell lines. Data are presented as mean of log RQ ± technical error of the replicates. **C)** Mammalian target of rapamycin (mTOR) (mTOR green, DAPI blue, magnification 20x, size-bar 40 µm). **D)** Ribosomal protein S6 kinase (S6P) (S6P red, DAPI blue, magnification 20x, size-bar 40 µm). **E)** Integrated density analysis of mTOR and PS6. Data presented as integrated density % ± SEM. Significant changes are marked as ★ ($P<0.05$).

5.2. LAM molecular background mapping: Deregulation of nuclear receptors, Wnt signalling, vascularization and miRNAs

To investigate whether estrogen and its receptor-regulated signalling play a role in LAM progression, molecular analysis of the cell lines was performed. A molecular map of LAM cell lines was built using different types of arrays for detection of protein and miRNA expression levels.

5.2.1. Nuclear receptor deregulation

To build a full molecular map and investigate whether crosstalk between mTOR and nuclear receptors occurs, a Human nuclear receptors TaqMan array was performed using pooled samples of four LAM cell lines and of two SMC lines, respectively. Nuclear receptors are crucial in the regulation of many cell processes e.g. metabolism, development, and reproduction [111]. The used array cards contain 90 nuclear receptors and incorporate four housekeeping genes: 18S ribosomal RNA (18S rRNA), Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Glucuronidase Beta (Gusb) and hypoxanthine guanine phosphoribosyl transferase (HPRT) [112] for better reproducibility. Average of the four housekeeping genes was used to normalize the results and to build the log RQ values of LAM compared to SMC controls. As LAM is dominantly a disease of women, many estrogen receptor levels were expected to be deregulated. Progesterone receptor (PGR) and estrogen related receptor gamma (ESRR***γ***) were significantly up regulated while estrogen receptor alpha (ER***α***) showed down regulation in LAM cell-lines compared to SMC controls. Also, PPAR gamma coactivator 1 beta (PPARGC1β) which regulates the transcriptional activity of ER***α***, was upregulated (Figure 8) [113]. Nuclear receptor deregulation is linked to mitochondrial activity, as up regulation of the active isoform of PGR increases mitochondrial membrane potential and cellular respiration. Also, ESRR***γ*** controls mitochondrial biogenesis and energy metabolism. Furthermore, PPARGC1β is also important, as its increased activity leads to increased number of mitochondria. Additionally, several retinoic acid receptors were significantly deregulated, including retinoic acid receptor beta (RARβ) [114]. RAR, in general, plays an important role in inhibition of proliferation and stimulation of cellular differentiation. To confirm that deregulation of the identified genes are consistent in all LAM cell lines, qRT-PCR was performed on individual samples. To test the correlation and hierarchy in interaction amongst different nuclear receptors, a general back propagation mathematical algorithm (artificial neural network or ANN) was used.

Changes in all RAR, PGR and nuclear receptor subfamily 5 group A member 2 (NR5A2) showed strong correlation in expression [110].

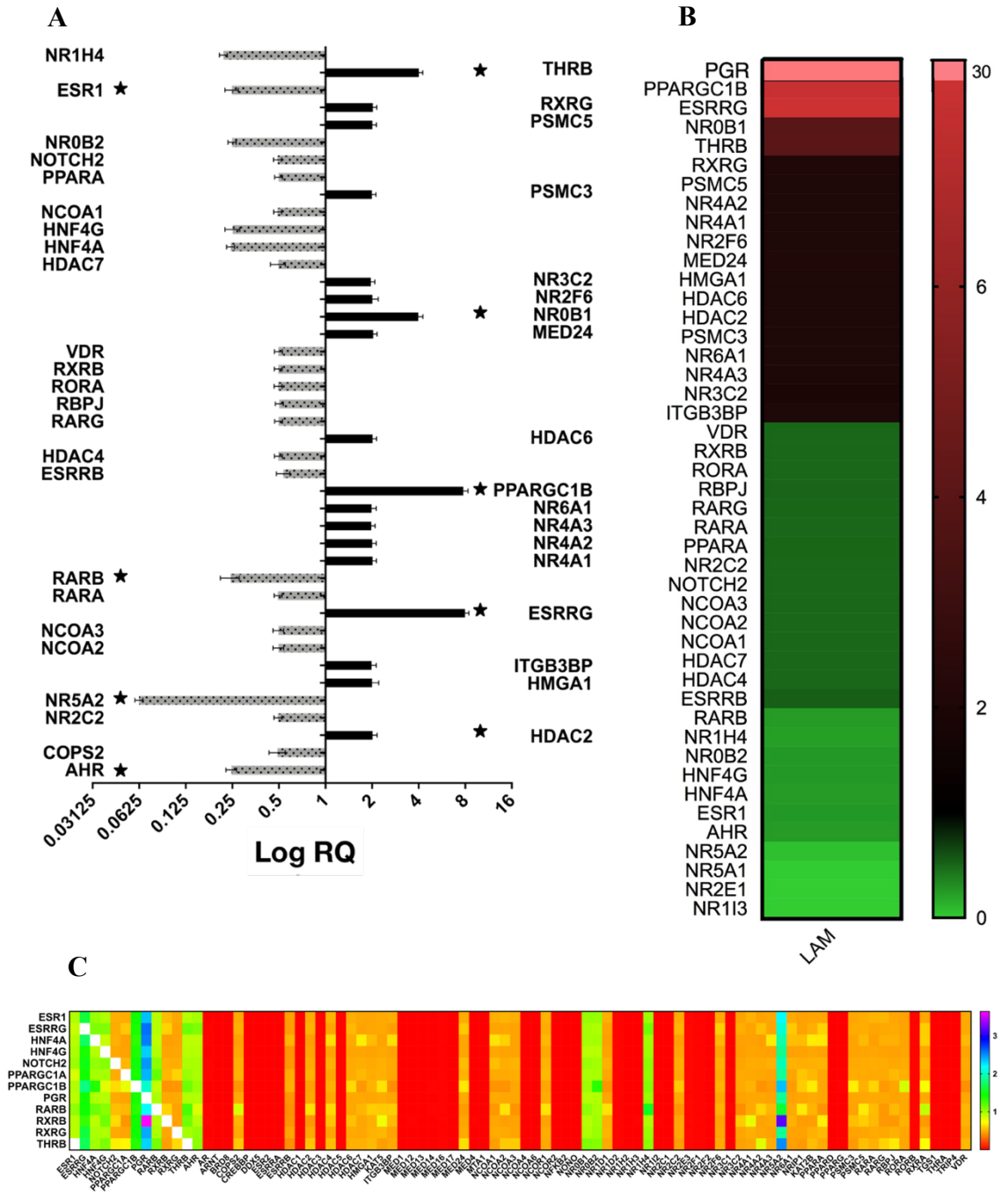


Figure 8. Molecular mapping of LAM cell lines compares to SMC. **A)** Nuclear receptor TaqMan arrays (data was generated from pooled samples of normal bronchial SMC controls n=2; or patient derived LAM cell lines n=4, respectively). data presented as LogRQ ± technical error of the replicates. **B)** Heat map of LogRQ values are shown. **C)** ANN analysis of the nuclear receptor arrays was performed to demonstrate hidden interactions amongst different nuclear receptors.

5.2.2. ***Evaluation of vascularisation markers***

Uncontrolled activation of mTOR pathway in LAM disease results from the loss of TSC1/2 and leads to continuous cell proliferation that requires higher energy state and an increase in the metabolism [115]. Higher energy demand and increased proliferation rate require better vascularization. Vascularization is driven by VEGF, VEGFR, and regulated by HIF1α [116]. qRT-PCR results from LAM patient-derived cell lines showed significant upregulation of the above genes (Figure 9A-B).

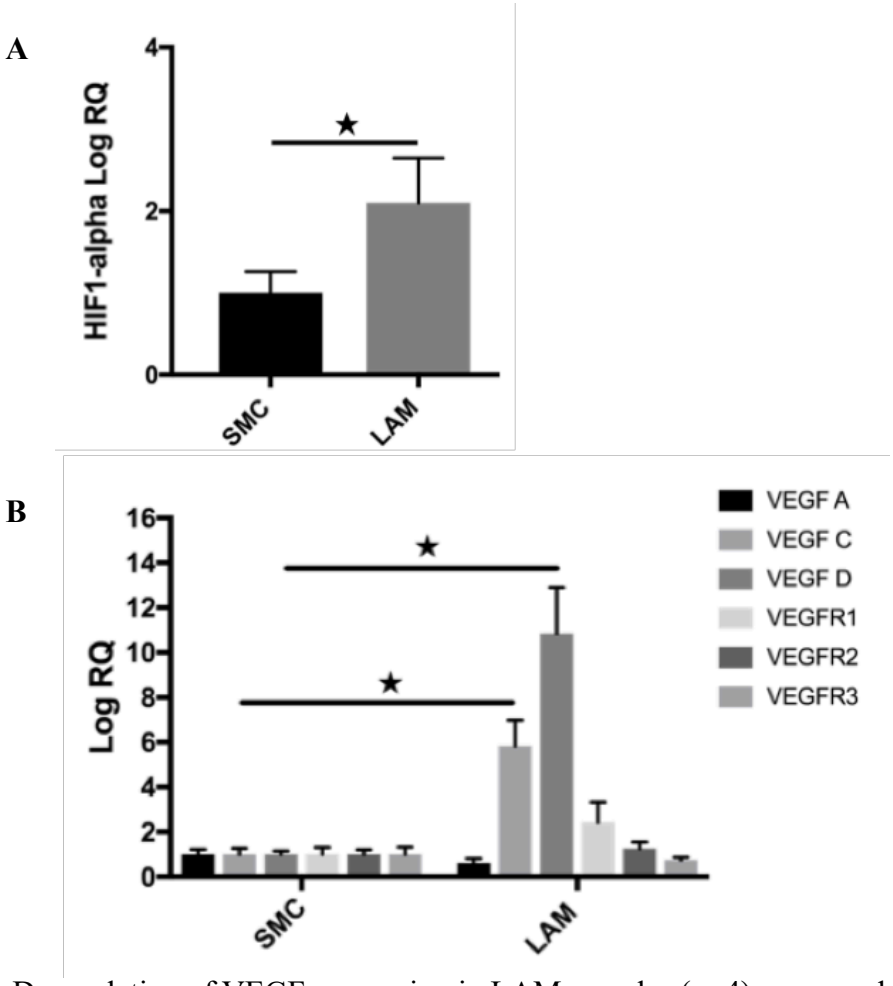


Figure 9. Deregulation of VEGF expression in LAM samples (n=4) compared to normal SMC (n=2). qRT-PCR analysis of genes affecting angiogenesis were performed and β-actin was used as inner control. Data are presented as mean of log RQ ± SEM. Significant changes are marked as ★ ($P<0.05$).

Full angiogenesis protein arrays were also performed on individual LAM cell lines and supported that not only at message levels but also at protein levels VEGF is upregulated (Figure 10A and 10B). The angiogenesis arrays have additionally revealed increased levels of C-X-C motif chemokine ligand 16 (CXCL16), a regulator of cellular invasion in lung cancer, and thrombospondin 1 (TSP1), an inhibitor of mitochondrial biogenesis [117]. ANN analysis of the angiogenesis array data revealed a strong association of CXCL16 and TSP1 with fibroblast growth factor (FGF), Endothelin1, SerpinE1 and VEGFC levels (Figure 10C).

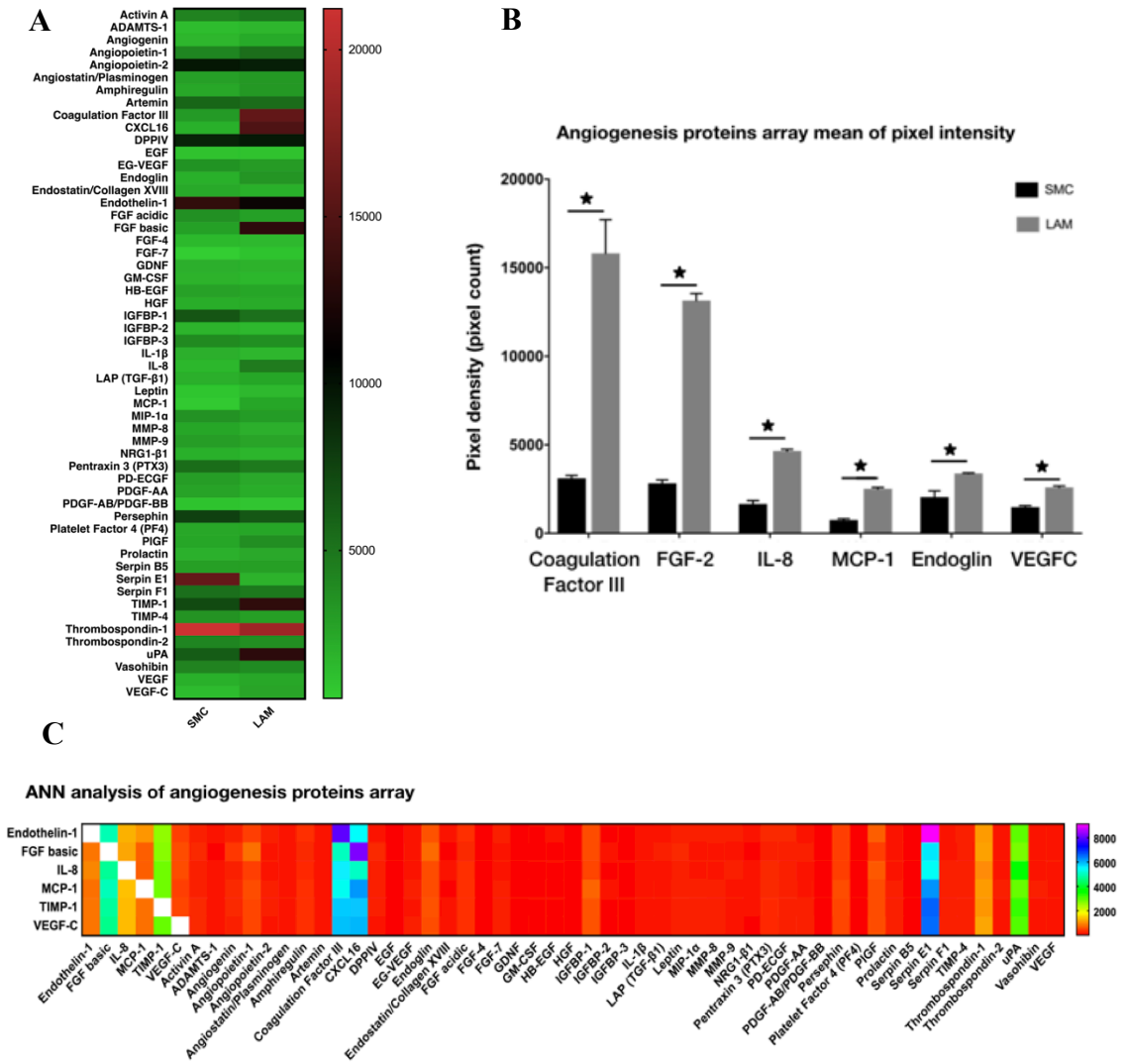


Figure 10. Angiogenesis protein arrays (data was generated from normal bronchial SMC controls (n=2); and patient derived LAM cell lines (n=4)). **A)** Heat map of angiogenesis protein array. The figure presents mean of pixel intensity. **B)** Angiogenesis array results presented as mean of pixel intensity ± SEM. Significant changes are marked as ★ ($P<0.05$). **C)** ANN analysis of angiogenesis protein interaction hierarchy.

5.2.3. Regulation of LAM disease genes by miRNA

miRNAs are crucial factors in the regulation of gene expression. To complete the molecular mapping of LAM cell lines, a Nanostring miRNA chip was used to identify the miRNA expression levels in the four LAM cell lines and the two SMC control cell lines. Using the Nsolver interface, negative controls were used to calculate the threshold for miRNA detection and results were normalized based on the top 100 miRNAs. Out of 750 more than 150 miRNAs were de-regulated. Both the tumour suppressor miR125b-5p [118] and the low density lipid oxidation induced autophagy regulator miR155-5p [119] were significantly downregulated compared to the control cell-line. While the apoptosis inducer miR-15b-5p [119] was downregulated, the cell proliferation and survival inducer miR-199a/b-3p [119] had increased copy numbers in LAM samples (Figure11).

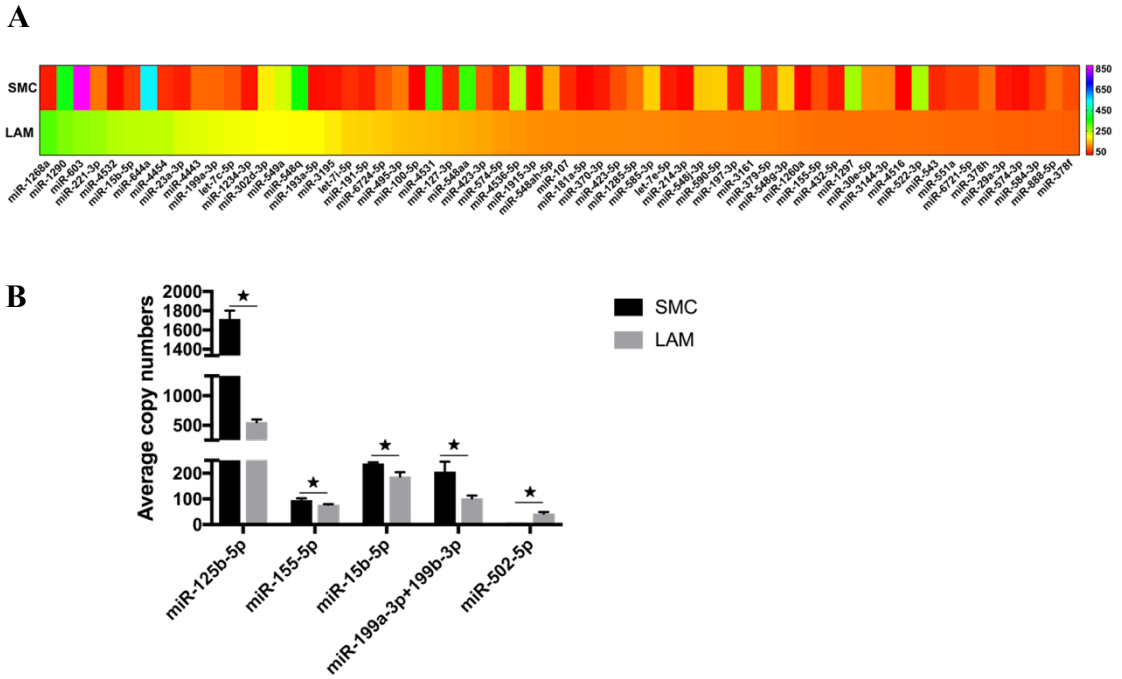


Figure 11. Analysis of 798 miRNA absolute copy numbers by Nanostring. miRNA copy numbers detected by Nanostring in pooled LAM (n=4) and pooled, normal, bronchial SMC (n=2) samples. **A)** The heat map represents the most deregulated 141 miRNAs in LAM samples compared to normal bronchial SMC controls. **B)** Copy number differences of specific miRNA-s that are involved in mitochondrial biogenesis detected after Nsolver analysis were further analysed in individual cell lines (normal bronchial SMCs (n=2) and LAM (n=4)). Data is presented as average copy number ± SEM, significant changes are marked as ★ ($P<0.05$).

In summary, the initial data suggested mitochondrial malfunction in LAM. To investigate this possibility, further studies were performed with a more specific focus on mitochondrial health.

5.3. Mitochondrial dysfunction in LAM

Studies of nuclear receptor and angiogenesis arrays revealed a significant upregulation of PPARGC1β expression. PPARGC1β is a key player in constitutive non-adrenergic-mediated mitochondrial biogenesis and is linked to this process via increased basal oxygen consumption, fat oxidation, non-oxidative glucose metabolism, and regulation of energy expenditure. Also, PPARGC1β is connected to NRF1 [85], a main regulator of the mitochondrial biogenesis. To investigate whether deregulation of PPARGC1β expression affected mitochondrial morphology, all four LAM cell lines and normal SMC control lines were subjected to electron microscopy. LAM cell mitochondria appeared denser, darker and abnormal compared to the normal SMC controls. There were also several empty looking vesicles in LAM cells which are absent in the normal SMC controls (Figure 12).

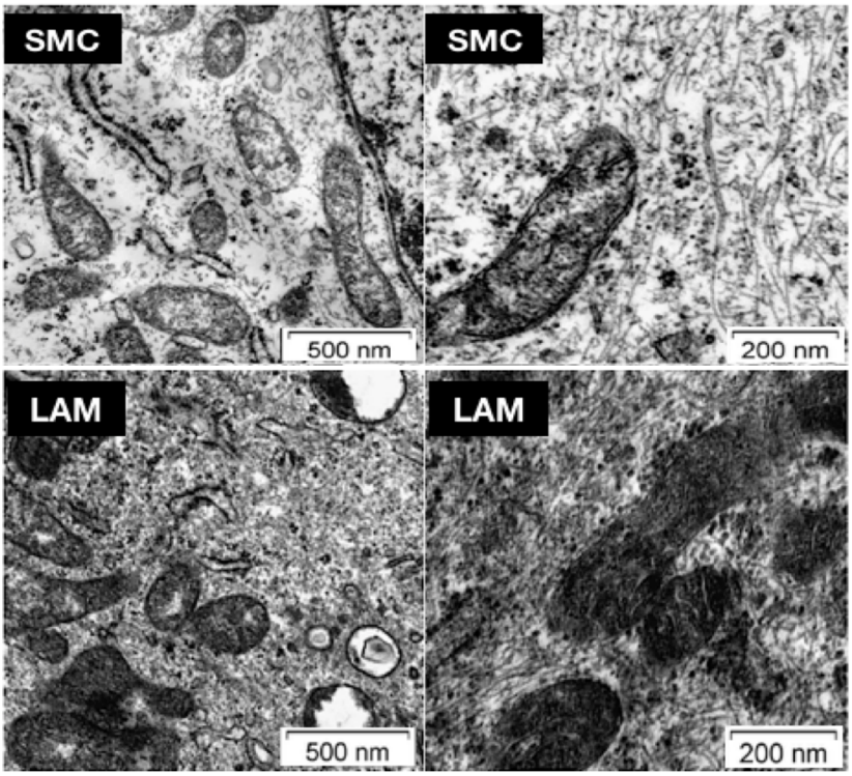


Figure 12. Mitochondria morphological alteration in LAM Cells. Electron microscopy of mitochondria in LAM cells and normal SMC controls (scale bar 500nm and scale 200nm, respectively).

To investigate the link between mitochondrial dysfunction and nuclear receptors, a qRT-PCR was performed to study mitochondrial gene expression. qRT-PCR revealed up-regulation of NRF1, TFAM, Cyto C and COX4 (Figure 13A). NRF1 protein plays a major role in cellular growth, respiration, mitochondrial DNA transcription, and also activates TFAM. Expression of TFAM, a gene that encodes a protein critical in both mitochondrial DNA repair and replication, was twice as high in LAM, than in control SMC samples. Cyto C and Cox 4, important components of the electron transport chain and respiratory chain showed a significant increase in LAM compared to normal SMC. Earlier studies found a connection between genes regulating mitochondrial genome transcription and replication e.g. TFAM and NRF1 and upregulation of electron transport chain and respiration proteins e.g. Cyto C and COX4 [120]. To study the impact of mitochondrial gene deregulation on mitochondrial metabolic activity and respiration; first, a flow cytometric analysis of the mitochondrial membrane potential was performed by adding RH-123 (a cell-permeable, cationic, green-fluorescent dye where rate of fluorescence decay is proportional to mitochondrial membrane potential) to the cell cultures. Higher membrane activity in LAM compared to SMC (Figure 13B) was detected using this method.

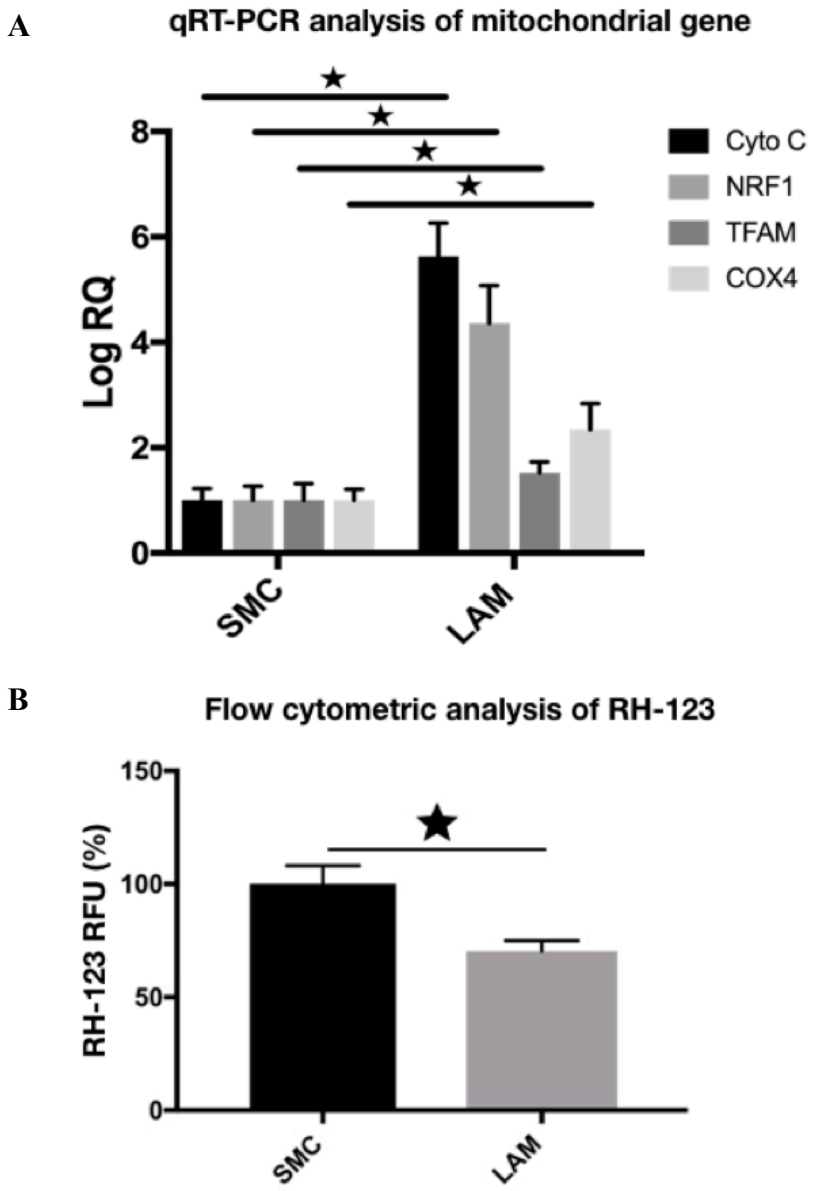


Figure 13. Altered function of mitochondria in LAM cells. **A)** qRT-PCR analysis of mitochondrial gene expression in individual LAM cell lines (n=4) compared to normal bronchial SMC controls (n=2). Data are presented as mean log RQ ± SEM and significant changes are marked as ★ ($P<0.05$). **B)** Flow cytometric analysis of RH-123 fluorescence intensity in individual LAM cell lines (n=4) compared to normal bronchial SMC controls (n=2). Data are presented as mean RFU ± SEM, significant changes are marked as ★ ($P<0.05$).

To investigate mitochondrial activity further, the Oroboros technique was used to measure oxygen consumption of LAM cells and control SMCs. Results revealed anaerobic respiration in LAM cells compared to normal SMCs (Figure 14). To measure both the oxygen consumption and glycolysis, SeaHorse XF96 analysis was also

performed (Figure 15).

Both SeaHorse XF96 and Oroboros confirmed lower oxygen consumption in LAM cells, additionally Seahorse XF96 analysis revealed higher glycolysis rate in LAM cells ("Warburg effect").

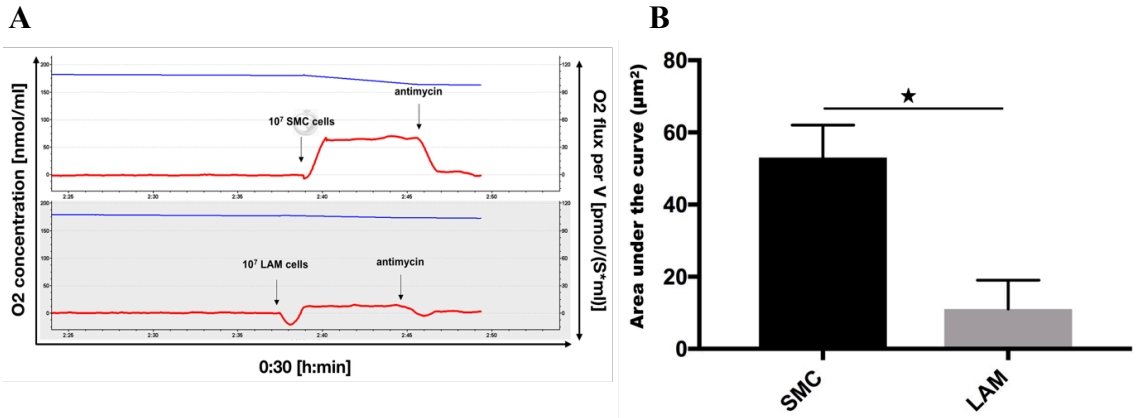


Figure 14. Measurements of mitochondrial activity. **A)** Oxygen consumption rate of mitochondria measured using Oroboros (blue line=oxygen concentration, red line= oxygen flux per Volume); **B)** Quantification of oxygen consumption (area under the curve).

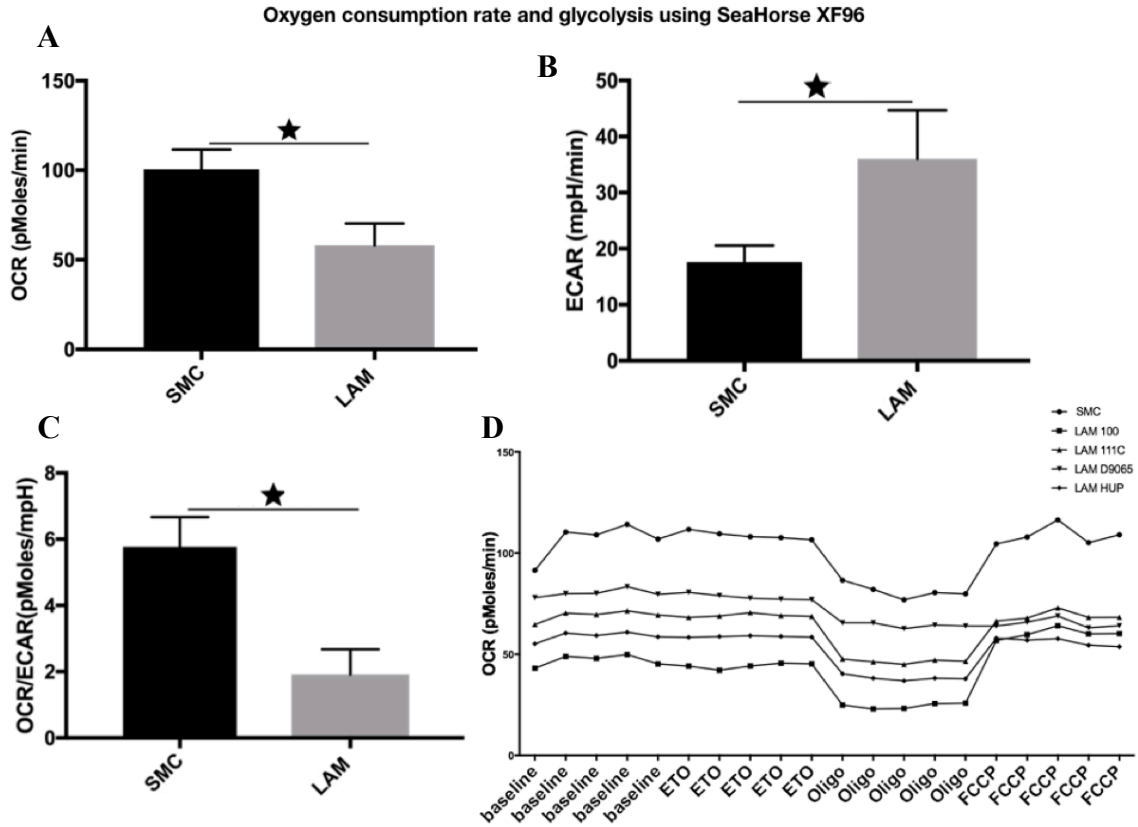


Figure 15. Oxygen consumption rate and glycolysis was measured by SeaHorse XF96 in individual LAM (n=4) cell lines and normal SMC control cells (n=2). Representative OCR and ECAR data are presented as mean ± SEM, ★ ($P<0.05$).

The “Warburg effect” during respiration results in higher amount of steady-state production of reactive oxygen species (ROS) and decrease in antioxidative capacity in the affected cells resulting in mitochondrial damage [88]. The cell in the state of elevated ROS production enhances its ROS scavenging system via oxidation of NADPH and thioredoxin reductase (TrxR). Normally, antioxidant matrix NADPH reductases, together with glutathione reductases and TrxRs [28] can all go on to generate H_2O_2 by leaking electrons from their reduced flavoprotein to O_2. Generation of this net mitochondrial ROS spill-over can cause oxidative injury and can critically damage mitochondria. Trx2–TrxR2 system has been reported to be an anti-angiogenic target of auranofin, a redox enzyme inhibitor gold complex [28]. The high affinity of auranofin for thiol and selenol groups and through the inhibition of redox enzymes such as TrxR can modify the redox balance in mitochondria. To investigate whether this is the case in LAM cells, a TrxR activity assay was performed and TrxR activity in LAM was significantly higher compared to SMC. This finding supports the theory that TrxR can leak electrons causing increased ROS production. This process could be used as a therapeutic target to restore normal mitochondrial function (Figure 16) [121].

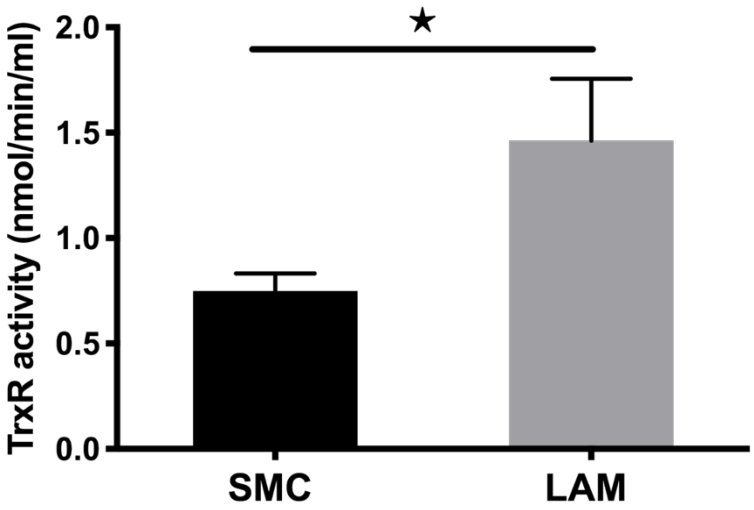


Figure 16. TrxR activity measured in individual LAM (n=4) and normal SMC control (n=2) samples. Data are TrxR activity is presented as mean of nmol.min/ml ± SEM, significant changes are marked as ★ ($P<0.05$).

5.4. *Mitochondria as a potential therapeutic target in LAM*

To attempt to heal mitochondrial dysfunction in LAM we used a novel synthetic flavonoid, Proxison (7-decyl-3-hydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone). Proxison is a potent antioxidant, which is rapidly incorporated into cells and can access the mitochondrial compartment [96]. The compound shares the backbone structure with myricetin, has high reactivity towards free radicals, and with its strategically placed lipophilic chain targets mitochondria to provide effective protection in the biological domain [122]. Proxison is capable of quenching a wide range of free radical species via an electron transfer/H-atom donation mechanism and efficiently protects cells against oxidative stress including preservation of membrane integrity and mitochondrial metabolic activity, reduction in lipid peroxidation and DNA damage, and the overall ability to retain cell viability and morphology [96]. To test the effect of Proxison, both normal SMCs and LAM cell lines were treated with Proxison. At first experiments were performed to confirm the lack of acute toxicity and inability to induce apoptosis in normal SMCs and LAM cell lines (Figure 17).

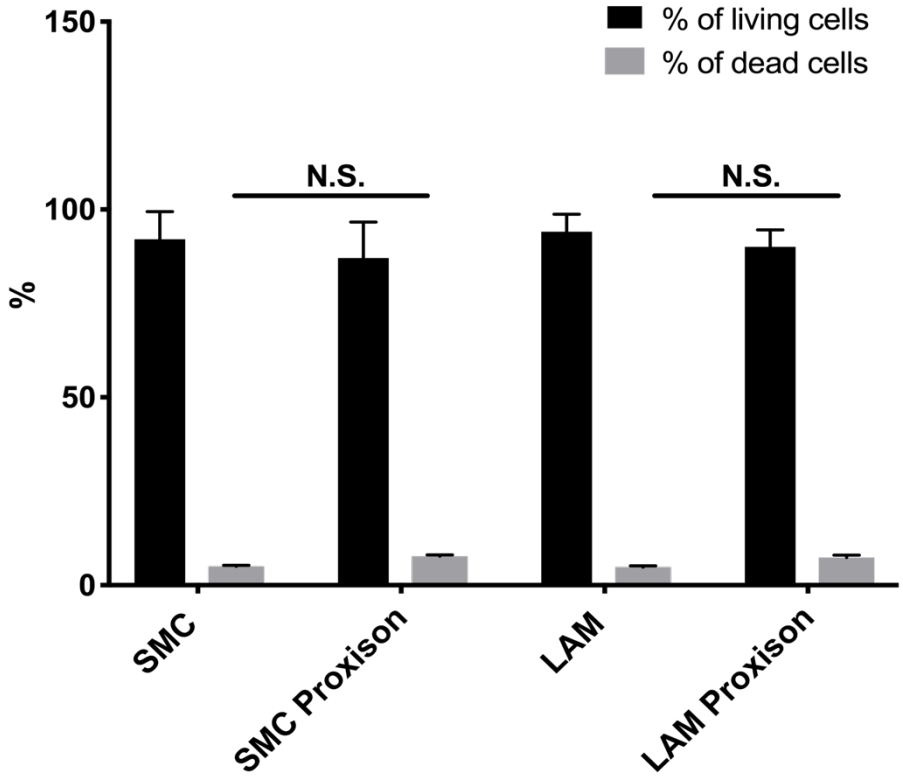


Figure 17. Proxison is not toxic in cell cultures. After Proxison (3 μM, 1 h) treatment viability of normal SMC (n=2) and LAM cell lines (n=4) were determined using propidium iodide (PI) (500 nM) then fluorescence intensity was analysed by flow cytometry. Data are presented as percentage of viability in 10,000 cells ± SEM.

Remarkably, rapidly after Proxison treatment, improvement of the mitochondrial cristae and inner membrane morphology were visible using electron microscopy (Figure 18).

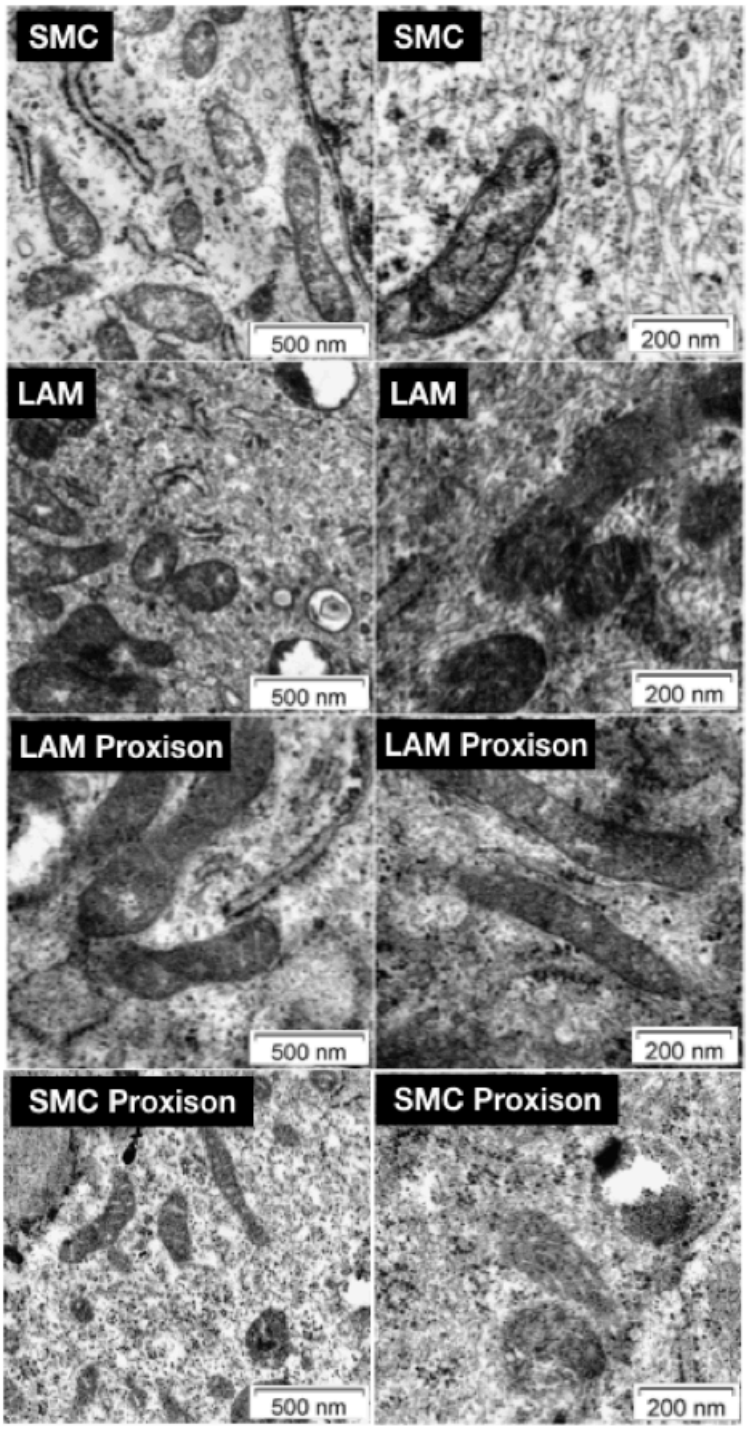


Figure 18. Proxison normalizes mitochondrial morphology and function in LAM cells. Representative morphological changes in the mitochondria of LAM cell lines following Proxison treatment. Electron microscopy of mitochondria of untreated and Proxison (3 μM, 1h) treated LAM cells and normal SMC control cells (scale bars are 500 nm and 200 nm, respectively).

Also, rapid improvement was detected in expression of mitochondrial health markers Cyto C, NRF1, TFAM and Cox4 genes using qRT-PCR following 1 hr treatment with Proxison (Figure 19).

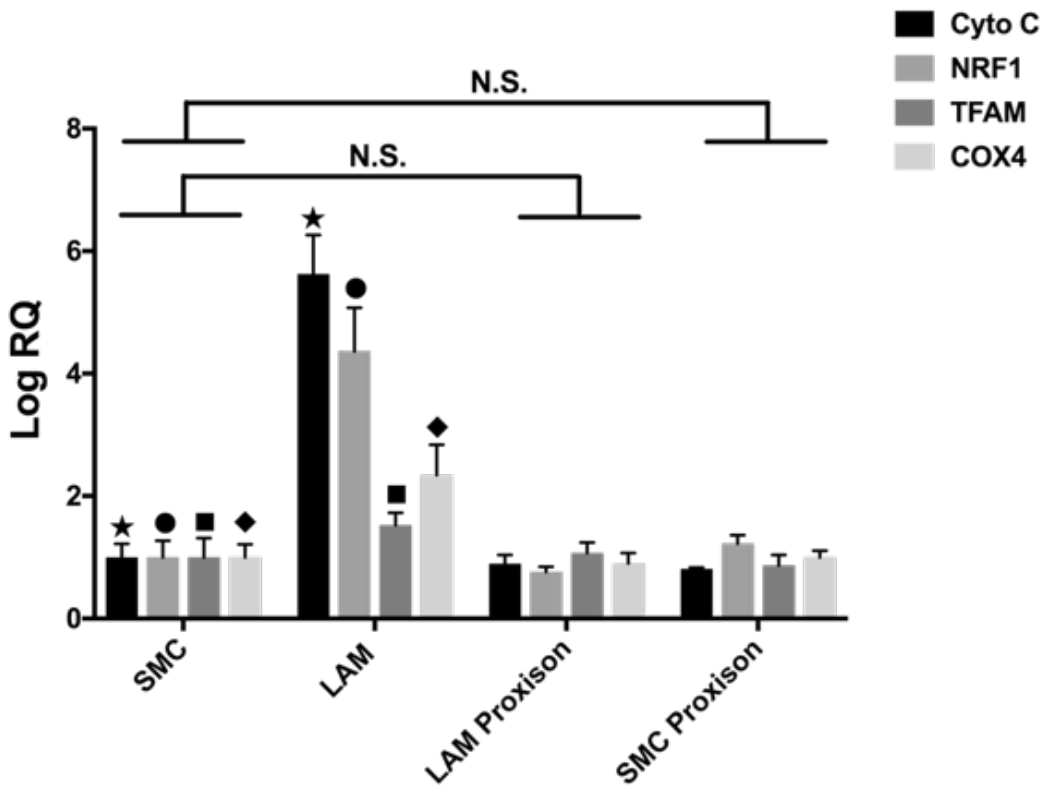


Figure 19. qRT-PCR analysis of mitochondrial gene expression in untreated and Proxison (3 µM, 1 h) treated LAM cell lines (n=4) compared to normal SMC controls (n=2); Data are presented as mean log RQ ± SEM and significant changes are marked as ★ (P<0.0001), ● (P<0.001), ◆ (P<0.01) and ■ (P<0.05).

Apart from the promising improvement in mitochondrial gene expressions, Proxison treatment had an impact on mitochondrial activity and membrane potential measured by RH-123 using both fluorescent microscopy and flow cytometry (Figure 20).

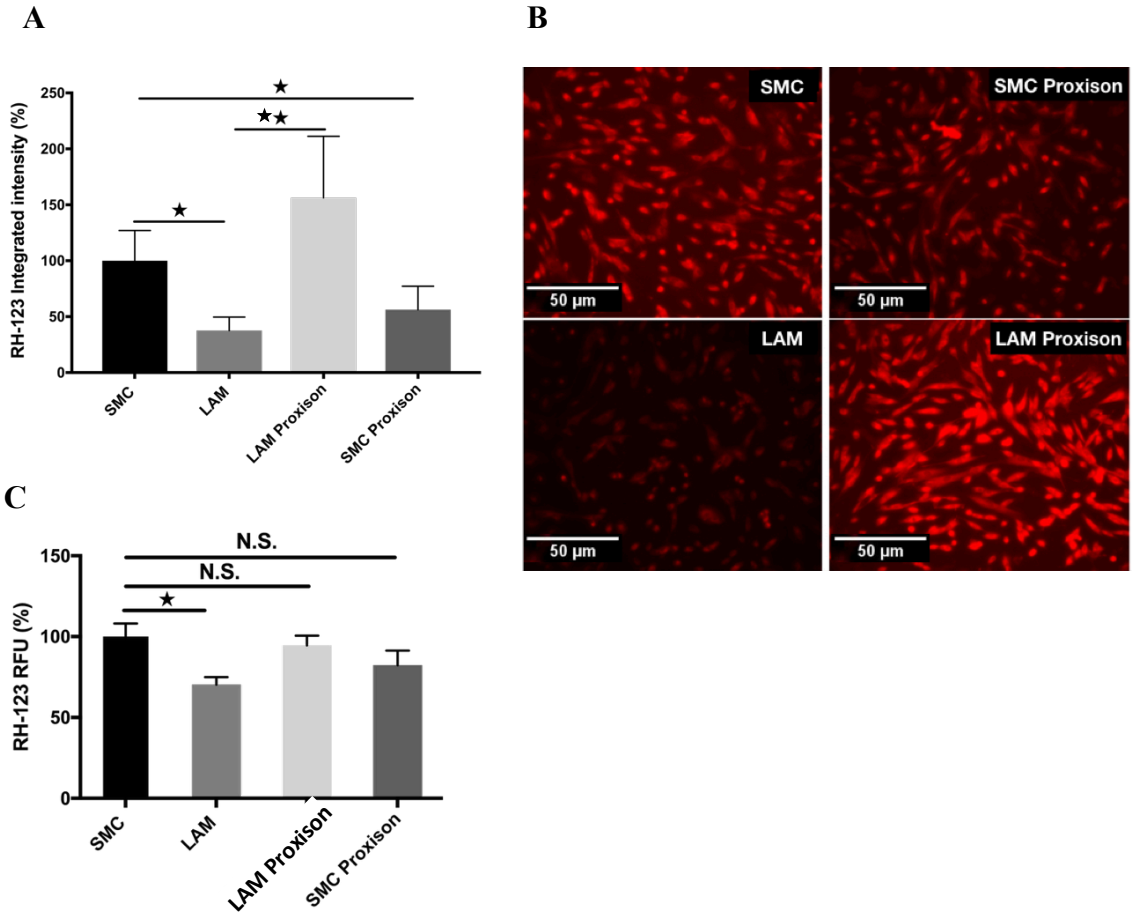


Figure 20. Membrane potential in Proxison treated LAM cells. Proxison (3 µM, 1 h) treated normal SMC and LAM cells were incubated with 2.5 µM RH-123 then fluorescence was analysed by: **A and B)** Fluorescence microscope. Data are presented as integrated density % ± SEM; significant changes marked as ★($P<0.05$). **C)** Flow cytometry. Data are presented as mean of RFU ± SEM; significant changes marked as ★($P<0.05$), ★★($P<0.001$).

Additionally, Proxison restored TrxR activity to normal level and consequently normalised ROS scavenging ability (Figure 21).

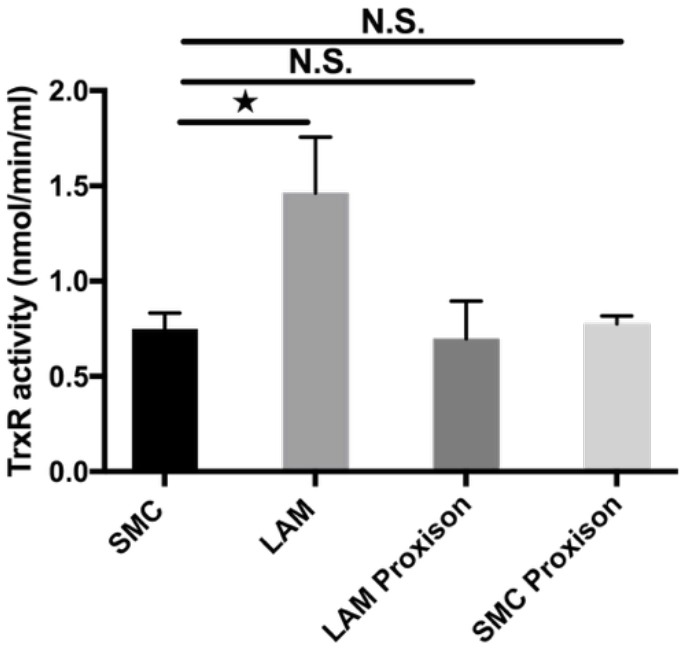
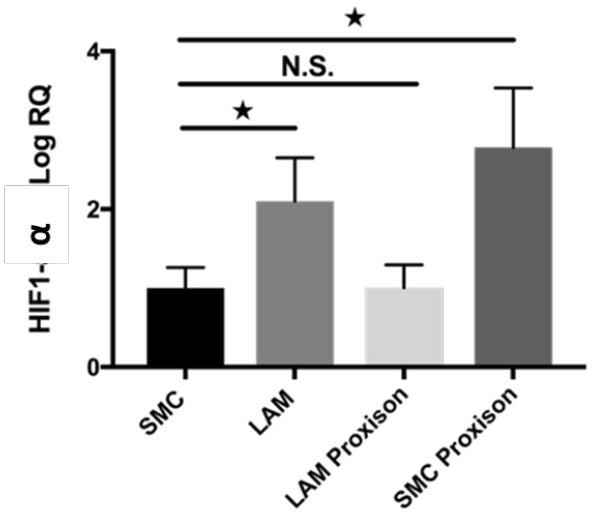


Figure 21. TrxR activity of Proxison (3 μM, 1h) treated LAM cell lines (n=4) compared to normal SMC controls (n=2). TrxR activity is presented as mean ± SEM and significant changes are marked as ★ ($P<0.05$).

Proxison treatment affected VEGF C, VEGF D and HIF1α expression, their levels falling back to normal in LAM cell lines (Figure 22).

A



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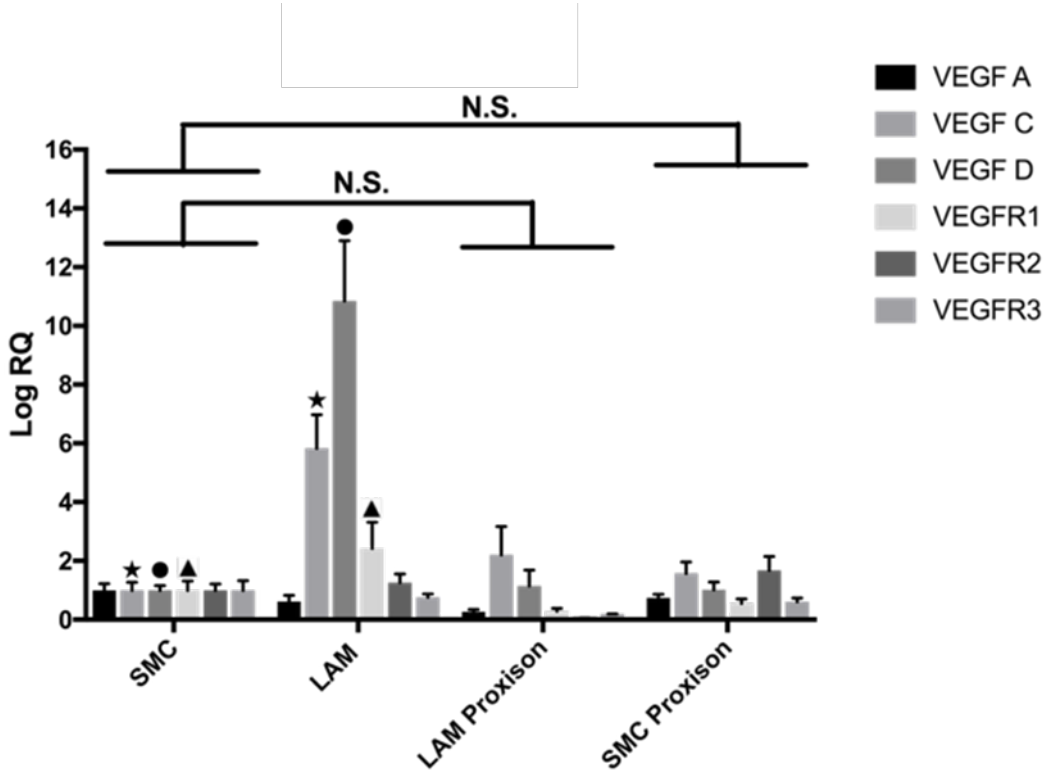
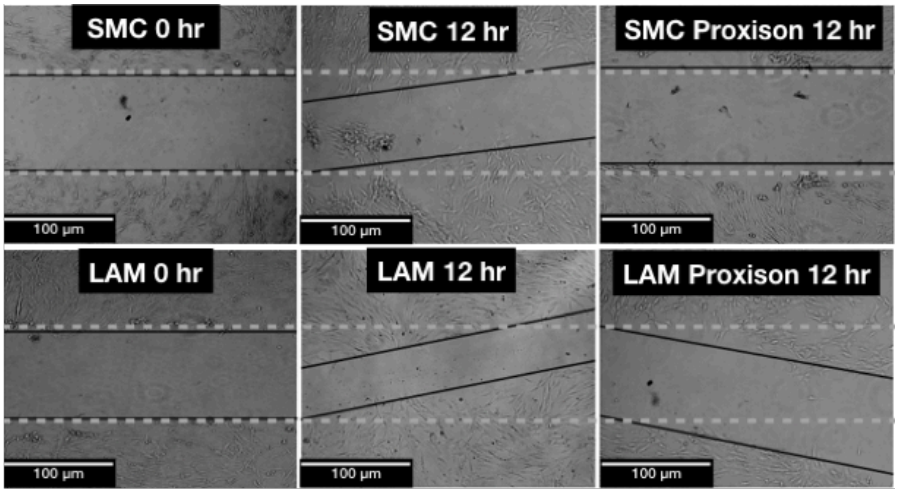


Figure 22. qRT-PCR analysis of angiogenesis related gene expression in untreated and Proxison (3 µM, 1 h) treated cell cultures (LAM n=4, normal SMC n=2). A) HIF1α and B) VEGF ligand and receptor mRNA levels. Data are presented as mean Log RQ ± SEM and significant changes are marked as ★, ● ($P<0.005$) and ▲ ($P<0.05$).

To test the effect of Proxison on cell proliferation, both scratch assays (Figure 23) and migration assays (Figure 24) were performed. Proxison was applied either as mono-treatment or in combination with Rapamycin. Proxison had an additive effect to Rapamycin, therefore reduction of the dosage of Rapamycin was necessary to keep gene expression and proliferation rate similar to untreated normal SMCs.

A



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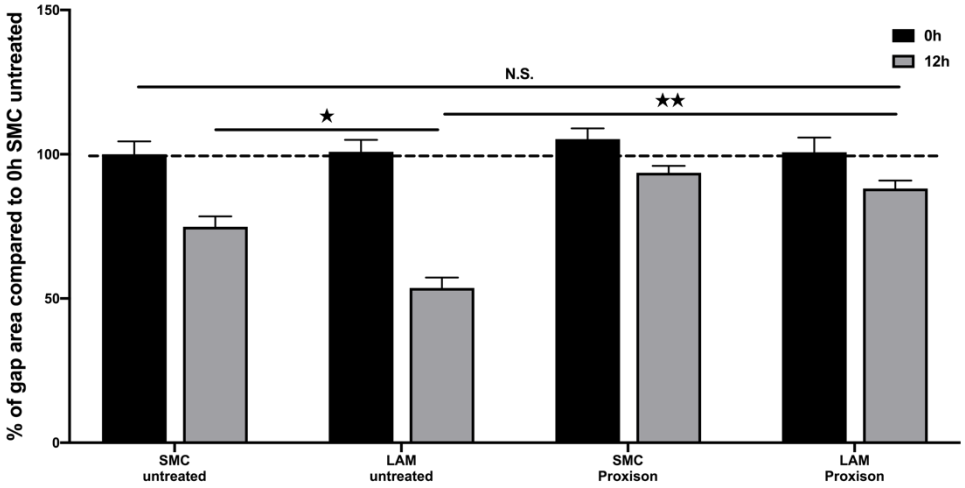


Figure 23. Proliferative capacity following Proxison treatment (n=3 technical repeats). **A)** Representative pictures of scratch assays in untreated and Proxison (3 µM, 1h) treated LAM cell lines (n=4) compared to normal SMC controls (n=2) after 12 h incubation. **B)** Data are presented as mean of cell growth (gap) area nm2 ± SEM, significant changes are marked as ★ ($P<0.05$) and ★★($P<0.001$).

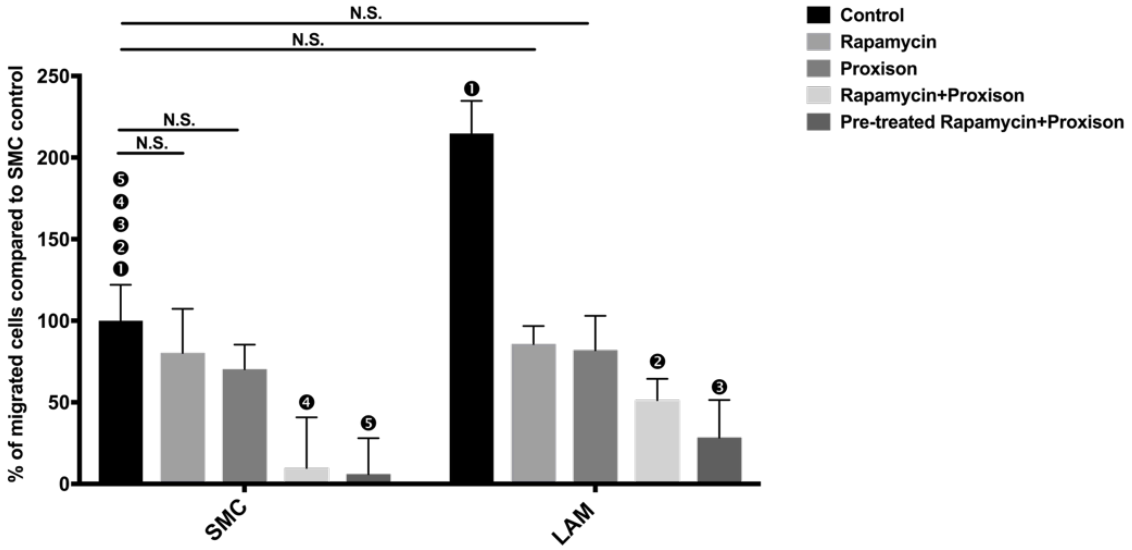


Figure 24. Migration capacity of LAM cell lines. LAM cell lines (n=2) and normal SMC (n=2) were treated with Rapamycin (20 nM, 24h), Proxison (3 μM, 24 h), Rapamycin (20 nM, 24h)+Proxison(3 μM, 24 h) and finally, cells were pre-treated with Rapamycin for 48h (20 nM/24h) then incubated with Proxison (3 μM, 24h). Images are presented as the number of cells migrated through the membrane to the lower side of the chamber and were stained with DAPI. Data are presented as percentage of migrated cells compared to normal SMC ± SEM and significant changes are marked as 1 ($P<0.0005$), 2 ($P<0.0045$), 3 ($P<0.0004$), 4 ($P<0.0001$) and 5 ($P<0.00001$).

5.5. Retinoic acid receptor as a potential therapeutic target in LAM

As the TaqMan array analysis detected reduction in RARβ expression, deregulation of vitamin A metabolism in LAM was presumed. qRT-PCR confirmed the initial findings as RARβ mRNA levels were reduced in individual LAM samples (Figure 25). To investigate the cause of RARβ downregulation, LAM cells were treated with retinoic acid (RA). Incubation of LAM cell lines with 2 μM RA restored RARβ levels to normal within 24 h (Figure 25).

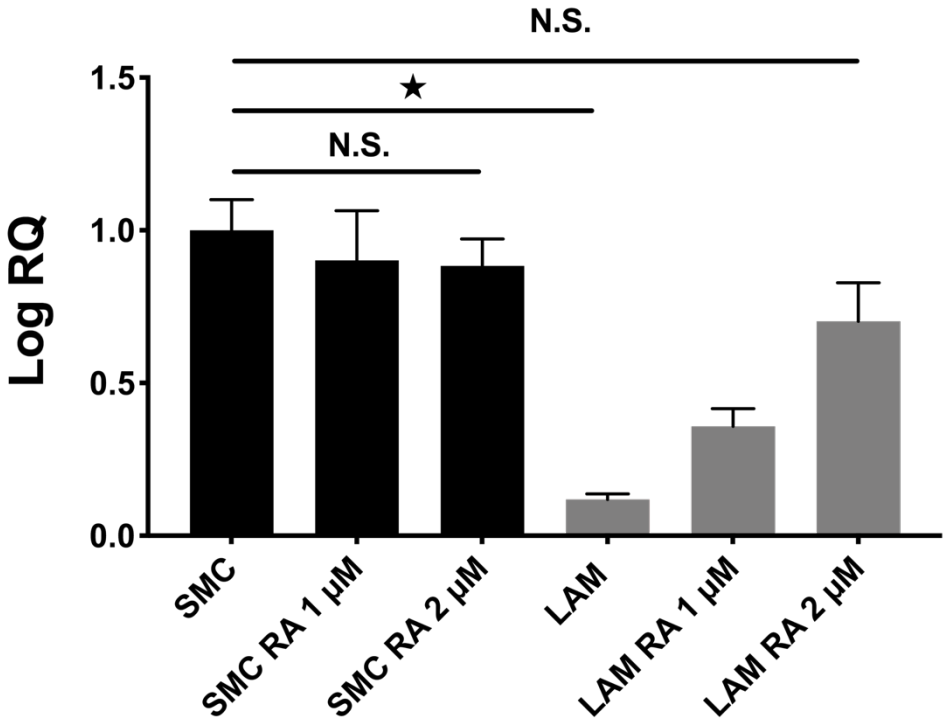


Figure 25. RA treatment effect. qRT-PCR analysis of RARβ gene expression in untreated and RA (1,2 μM, 24 h) treated cell cultures (LAM n=4, normal SMC n=2). Data are presented as mean RQ ± SEM and significant changes are marked as ★ (in all results significance was $P<0.05$).

As RA restored RARβ gene expression to normal, it suggested the existence of a different fault. To decipher the mechanism, the overall vitamin A metabolism was analysed. There are two main sources of vitamin A, retinol, from animal source and β-caroten from plant source. One of the 600 known naturally occurring plant derived carotenoids is Lutein. The oxidised form of retinol is RA. Both sources have their own metabolic pathways to reach their shared nuclear receptors, and they also share some metabolic enzymes (Figure 26).

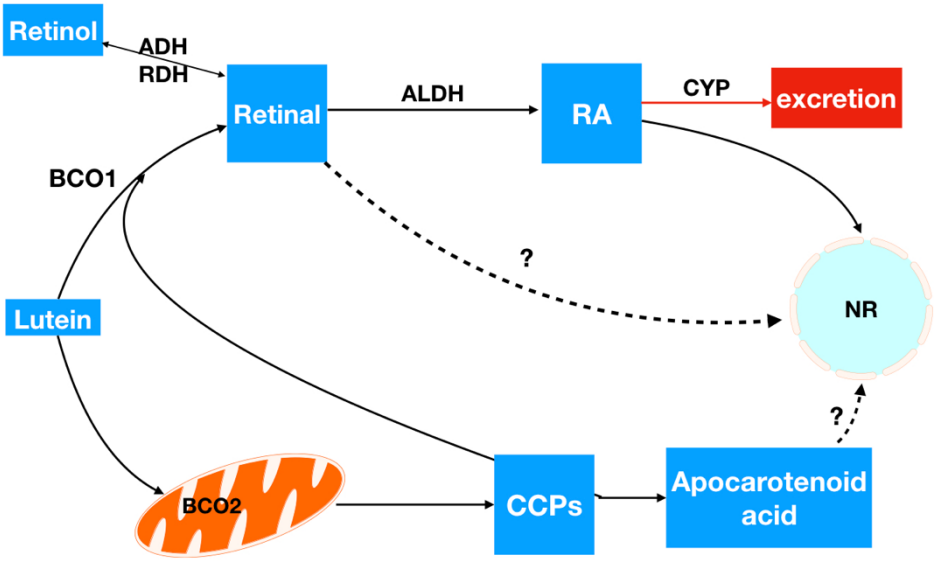


Figure 26. Summary of Lutein and RA metabolism

To study the reasons behind for RARβ deregulation, qRT-PCR was performed to test the relative quantity of enzymes involved in vitamin A metabolism. Alcohol dehydrogenase (ALDH1A1), β-carotene dehydrogenase 1 (BCO1) and β-carotene dehydrogenase 2 (BCO2) were significantly downregulated in LAM cell lines compared to levels measured in normal SMCs (Figure 27). Practically, LAM cells were lacking BCO2 and ALDH1. Meanwhile, retinol dehydrogenase (RDH) was significantly upregulated in LAM cell lines (Figure 27A and B). To investigate whether vitamin A metabolites affect enzyme levels, LAM cell lines and SMC controls were treated with RA and lutein. In contrast, Rapamycin induced a slight increase of BCO1 and RDH1 but had no effect on BCO2 and ALDH1 in LAM. Interestingly, while RA and lutein treatment restored the above mentioned enzymes close to normal levels, combination treatment with Rapamycin resulted in extreme increase in RDH1 and a complete loss of ALDH1 levels (Figure 27A and B). All Rapamycin, RA and lutein increased RARβ levels to normal or above but a

combination treatment with Rapamycin and lutein induced a five-fold increase in RARβ expression in control SMCs (Figure 27C).

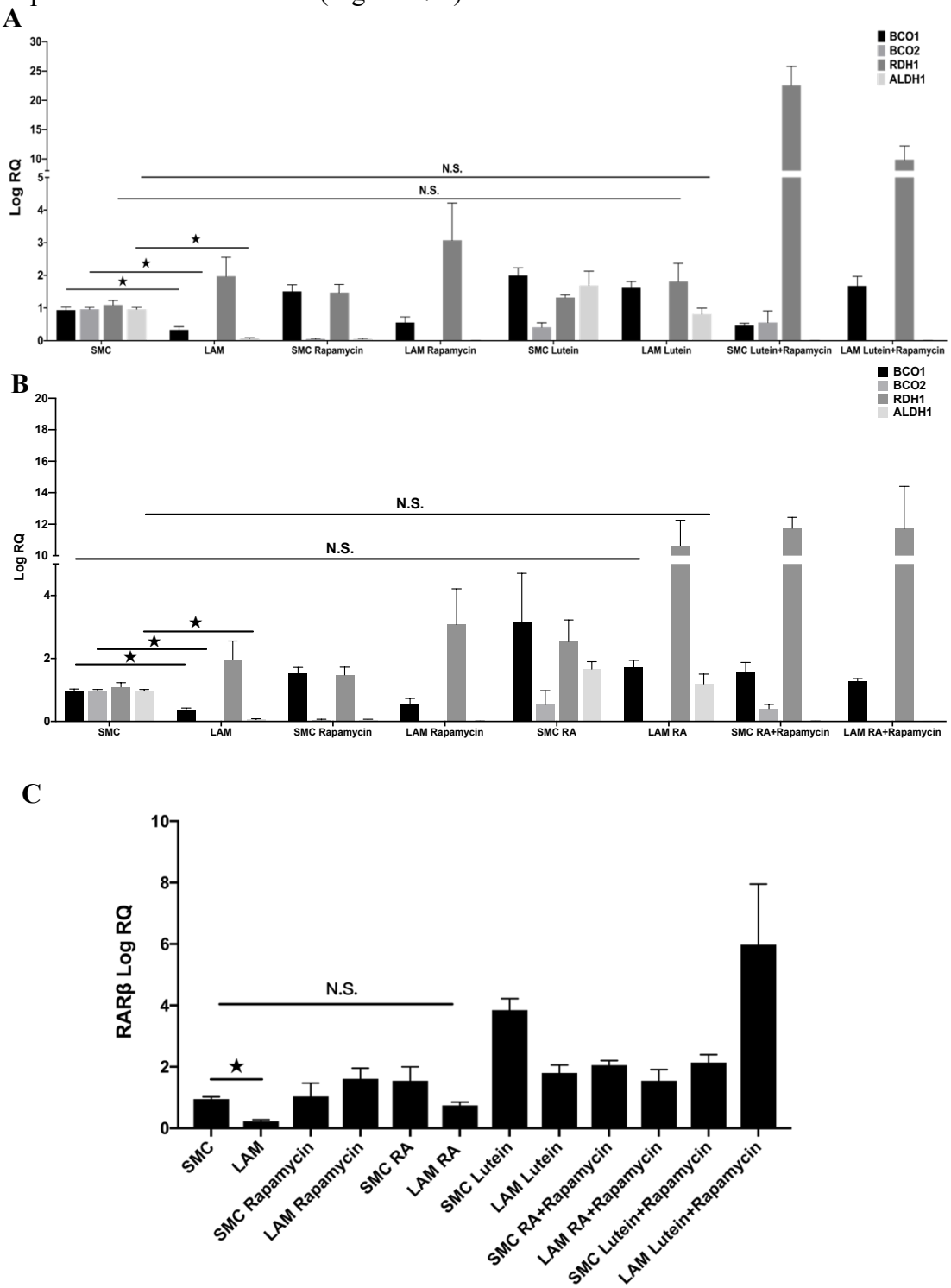
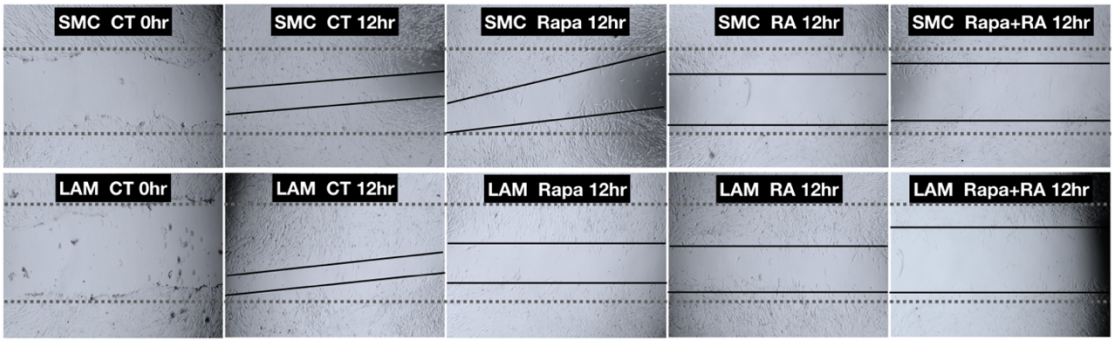


Figure 27. Effect of RA and Lutein on enzyme expressions of vitamin A metabolism. qRT-PCR of RARβ, RDH, ALDH, BCO1 and BCO2 enzymes expression in LAM (n=4) compared to normal SMC (n=2) after treatment with **A)** Lutein (100 µM, 24 h), Rapamycin (20 nM, 24h), Lutein (100 µM, 24 h)+Rapamycin (20 nM, 24h) and **B)** RA (2 µM, 24 h), Rapamycin (20 nM, 24h), RA (2 µM, 24 h)+Rapamycin (20 nM, 24h). **C)** The effect of RA, Lutein, Rapamycin, RA+Rapamycin and Lutein+Rapamycin on RARβ expression. Data presented as LogRQ ± SEM, significant changes are marked as ★ ($P<0.05$).

Scratch assay was used to assess the effect of RA treatment on cellular proliferation and migration capacity of LAM cells. Combination of RA with Rapamycin resulted in lower proliferation and migration capacity, than detected in Rapamycin monotherapy (Figure 28).

A



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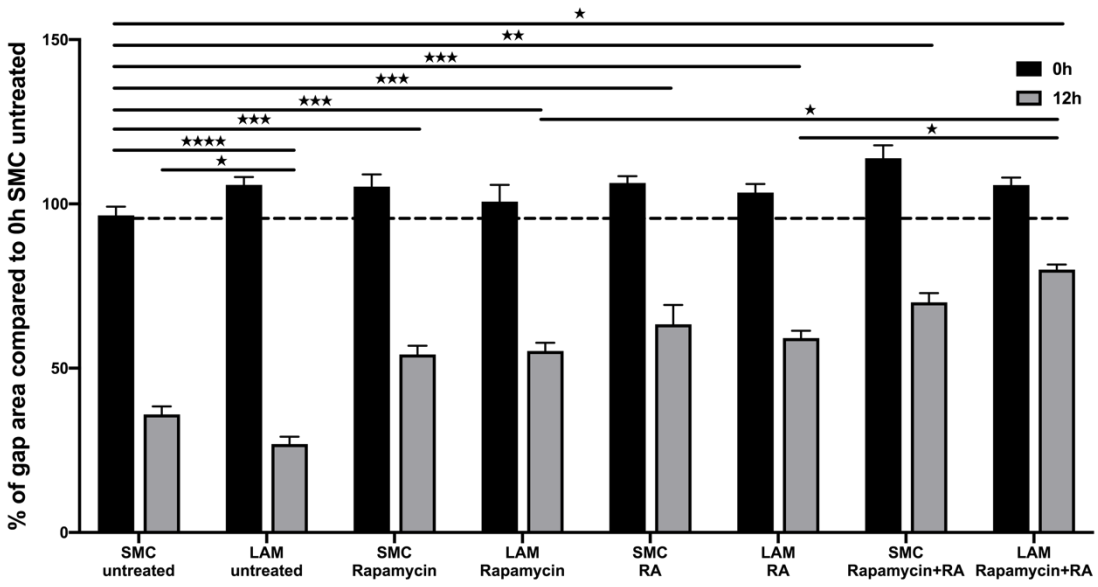

Figure 28. Proliferation capacity following RA treatment (n=3 technical repeats). **A)** Representative pictures of scratch assays in untreated, Rapamycin (20 nM, 24h), RA (2 µM, 24h) and RA (2 µM, 24 h)+Rapamycin (20 nM, 24h) treated LAM cell lines (n=4) compared to normal SMC controls (n=2) after 24 h incubation. **B)** Data are presented as mean of cell growth (gap) area nm^2 ± SEM, significant changes are marked as ★ ($P<0.05$), ★★ ($P<0.001$) and ★★★ ($P<0.0001$).

5.6. *Deregulation of WNT signalling in LAM*

As one of the more robust regulators of the mTOR pathway is Wnt signalling, individual human Wnt signalling TaqMan array plates were used to analyse the gene expression levels of molecules in the various Wnt signalling pathways. All four LAM patient derived cell lines and the two normal SMC cell lines were tested. Out of the investigated 92 genes, 43 genes were deregulated in LAM. 36 genes were significantly downregulated and 7 genes were upregulated (Figure 29). The downregulated genes included secreted extracellular inhibitors SFRP2, SFRP4 and DKK2. Earlier studies indicated inhibitory effect of SFRP4 on AMPK signalling pathway and mitochondrial depolarization. Also SFRP2 is regulated by AKT [21]. Based on the literature, loss of SFRP4 leads to aggressive cancers, epithelial mesenchymal transition (EMT), increased cell migration and deregulation of downstream signalling molecules within the Wnt signalling pathways. Downregulation of DKK2 that inhibits STAT5 signalling can also have severe consequences. STAT5 proteins are activated by a wide variety of hematopoietic and non-hematopoietic cytokines and growth factors, and critically regulate vital cellular functions such as proliferation, differentiation, and survival. The physiological importance of active STAT5 proteins is obvious in a large number of primary human tumours that have aberrant constitutive activation of these proteins, which significantly contributes to tumor cell survival and malignant progression of the disease [123]. DKK1, another extracellular inhibitor of the canonical Wnt pathway, however, was significantly upregulated, and based on the literature, DKK1 is known to promote migration and invasion in liver cancer via shifting canonical Wnt activation towards non-canonical Wnt pathway activity leading to increased inflammatory processes by activation of the NF-kB pathway [124]. DKK1 has just recently been recognised to play an important part in the pathophysiology of arterial wall. The upregulated Wnt5b, one of the 19 ligands of the Wnt signalling pathway, is known for its role in activation of PPARγ and induction of adipogenesis [53] as well as induction of tube formation by regulating the expression of Snail and Slug proteins via activation of both canonical and non-canonical Wnt signalling pathways. Wnt5b can also modulate mitochondrial activity via MCL1 and is known to promote cell motility and metastasis in various cancers [57]. The intracellular canonical Wnt signalling mediator, GSK3β - an important regulator of the mTOR pathway - was also upregulated [63]. Simple upregulation of GSK3β is not informative as its enzymatic activity strongly depends on its level of phosphorylation. Deregulated activity of GSK3β has been observed in many human pathologies e.g.

cancers and non-insulin-dependent diabetes mellitus (NIDDM) [125]. Phosphorylation of GSK3β can suppress its activity leading to β-catenin accumulation in the cytosol.

The accumulated β-catenin molecules then translocate to the nucleus and increase canonical Wnt target gene transcription. Suppression of GSK3β by AKT and mTOR pathway can shift Wnt signalling to increased non-canonical Wnt pathway activity that has also been associated with cancer progression [126]. Additionally, RHOU - a molecule that is involved in various cellular processes - was also upregulated. In *in vitro* experiments RHOU has oncogenic activity and promotes cancer cell invasion. Its increased expression and activity correlates with carcinogenesis in a variety of cancers [127].

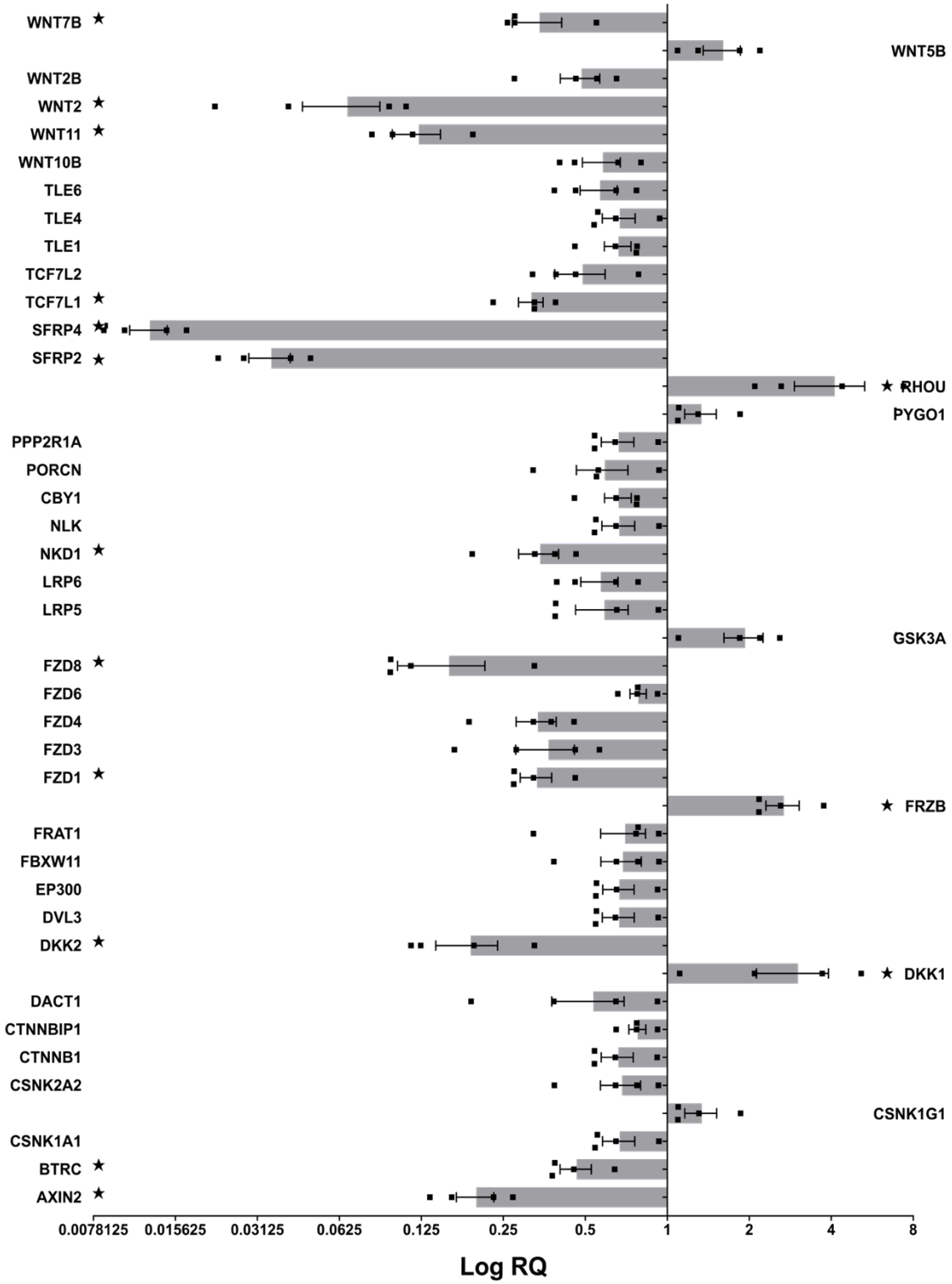

Figure 29. Molecular mapping of the Wnt pathway in LAM cell lines. Human Wnt signalling pathway TaqMan arrays of LAM cell lines (n=4) compared to normal SMC controls (n=2). Data presented as LogRQ ± SEM.

6. CONCLUSIONS

The rare disease LAM, presents a largely unmet medical need. Although recently animal models have started to emerge, fully human models of the disease are important to fully reveal the molecular background and to identify therapeutic targets. In the present work a great variety of molecular platforms were used to study LAM in patient derived cellular systems.

Based on the study, it was identified that uncontrolled proliferation of SMC-like LAM cells is just partly the consequence of TSC mutation and deregulation of the mTOR signalling pathway [65]. Mitochondrial dysfunction, deregulated vitamin A metabolism and altered Wnt signalling might also contribute to the disease [17].

Mitochondria are regulated via complex molecular pathways and in the absence of further mutation analysis it is still unclear whether simply TSC mutation can lead to mitochondrial deregulation and metabolic malfunction [87]. However, as in about 15% of all LAM cases have no TSC mutations, one can speculate that mutations in mitochondrial or Wnt signalling pathway genes can also lead to similar symptoms. The present study identified many deregulated molecules including nuclear receptors of hormones such as estrogen, transcriptional coactivators such as PPARGC1α and PPARGC1β that together with peroxisome proliferator-activating receptor (PPAR) genes, estrogen-related receptor (ERR) genes and NRF-1 coordinate the energy metabolism [128]. The altered expression of the above genes substantiated the importance of investigating the role of mitochondria in LAM pathogenesis. To make the picture even more complex, regulators of vitamin A metabolism and the Wnt signalling pathway is also deregulated in LAM and provided further targets for studies of therapeutic intervention [53][57].

Based on the results, restoration of mitochondrial activity is a strong candidate in LAM therapy. A pre-clinical drug candidate, Proxison, was chosen for the test. With its very high antioxidative capacity and capabilities to normalize both the electron transport chain and membrane potential [96], Proxison appeared to be a “wonder drug” for correction of mitochondrial dysfunction. Many flavonoids - such as myricetin and quercetin - are known for their antioxidative activity specifically targeting mitochondria, but Proxison’s modified structure improved its antioxidative ability as became superior in healing

diseased mitochondria [96].
True to expectations, Proxison restored mitochondrial activity and corrected deregulated gene expressions while was no cytotoxic to cells. In fact, Proxison had more robust effect on cell functions than Rapamycin, the clinically approved drug for LAM treatment.

Molecular mapping of patient derived cell lines have also revealed deregulation of additional pathways that interact with mTOR. Previous studies have found that RAR and estrogen oppose the action of each other [129]. Furthermore that estrogen response element (ERE) and retinoic acid response element (RARE) colocalize in the genome and regulate the expression of shared target genes [42]. It was suggested that ER and RAR may compete for transcriptional activity or inhibit each other depending on the availability of their ligands. Since ERRG also binds to ERE, increase in its transcriptional activity may decrease the expression of RARs [42]. RA competes with estrogen to inhibit or trigger proliferation, respectively [42]. RA is an antitumor agent known to be anti-proliferative, pro-apoptotic and anti-metastatic. Treatment of LAM cell lines with RA restored normal levels of RARβ within 24 h of treatment [37]. RA not just restored RARβ mRNA expression to normal levels but also lead to reduced proliferation as well as cellular migration of LAM cells. Combination of RA with Rapamycin, however, have also raised some warning signs as gene expression of vitamin A metabolic enzymes were markedly deregulated in combination treatment.

Molecular mapping of the evolutionarily conserved Wnt signalling pathway has also identified some potential therapeutic targets. Deregulation of canonical Wnt pathway inhibitors DKK1 and DKK2, SFRP 2 and 4 are all associated with aggressive carcinogenesis and recently became therapeutic targets not just in carcinomas but in other diseases associated with inflammation and vascularization [59]. The upregulated ligand, Wnt5b and the signalling molecule GSK3β are also involved in mTOR signalling but further studies are needed to understand their specific roles in LAM development and progression.

Some factors of various signalling pathways and especially mitochondrial dysfunction that was exposed during the study can be used as potential therapeutic target in LAM. The effects of both Proxison and RA were particularly promising to reverse mitochondrial dysfunction and to halt uncontrolled proliferation, respectively. As combination of Proxison and/or RA with Rapamycin could even decrease the clinically

applied dosage of Rapamycin, the current results could almost immediately have a direct effect on Rapamycin dependency of LAM patients leading to reduced severity of Rapamycin side effects (Figure 30).

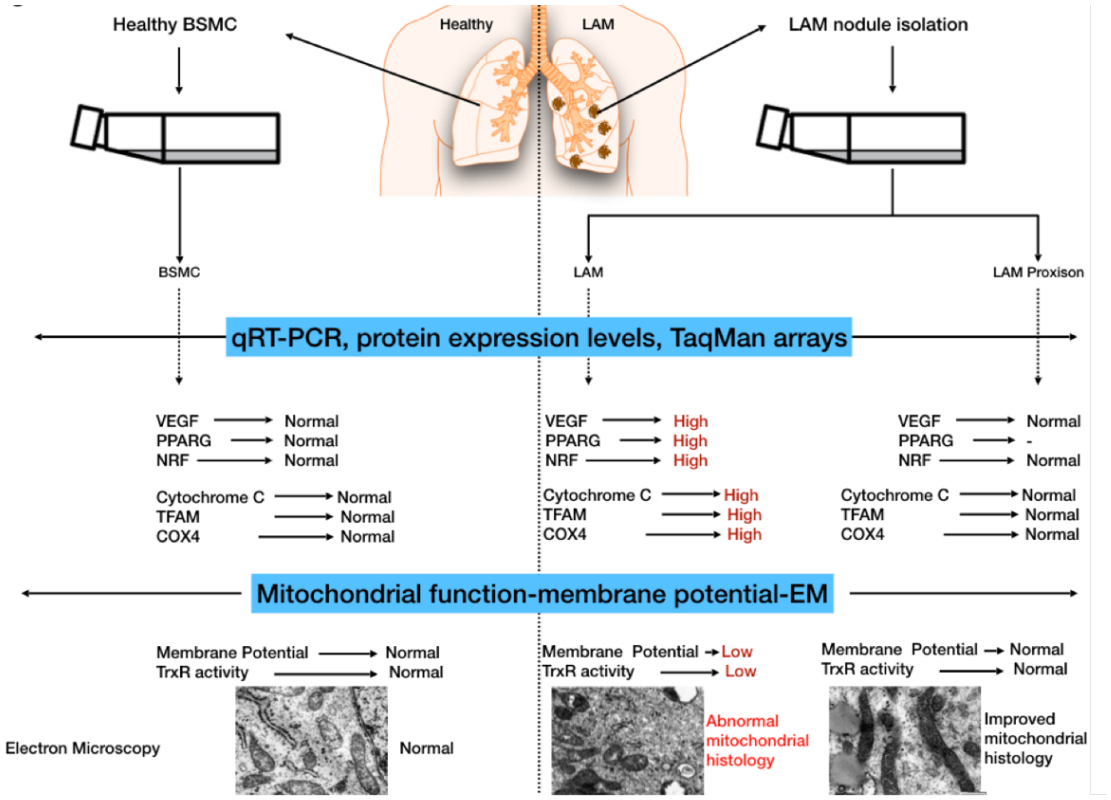

Figure 30A. Summary of LAM characteristics. The summary of pathomechanism and mitochondrial dysfunction study of LAM.

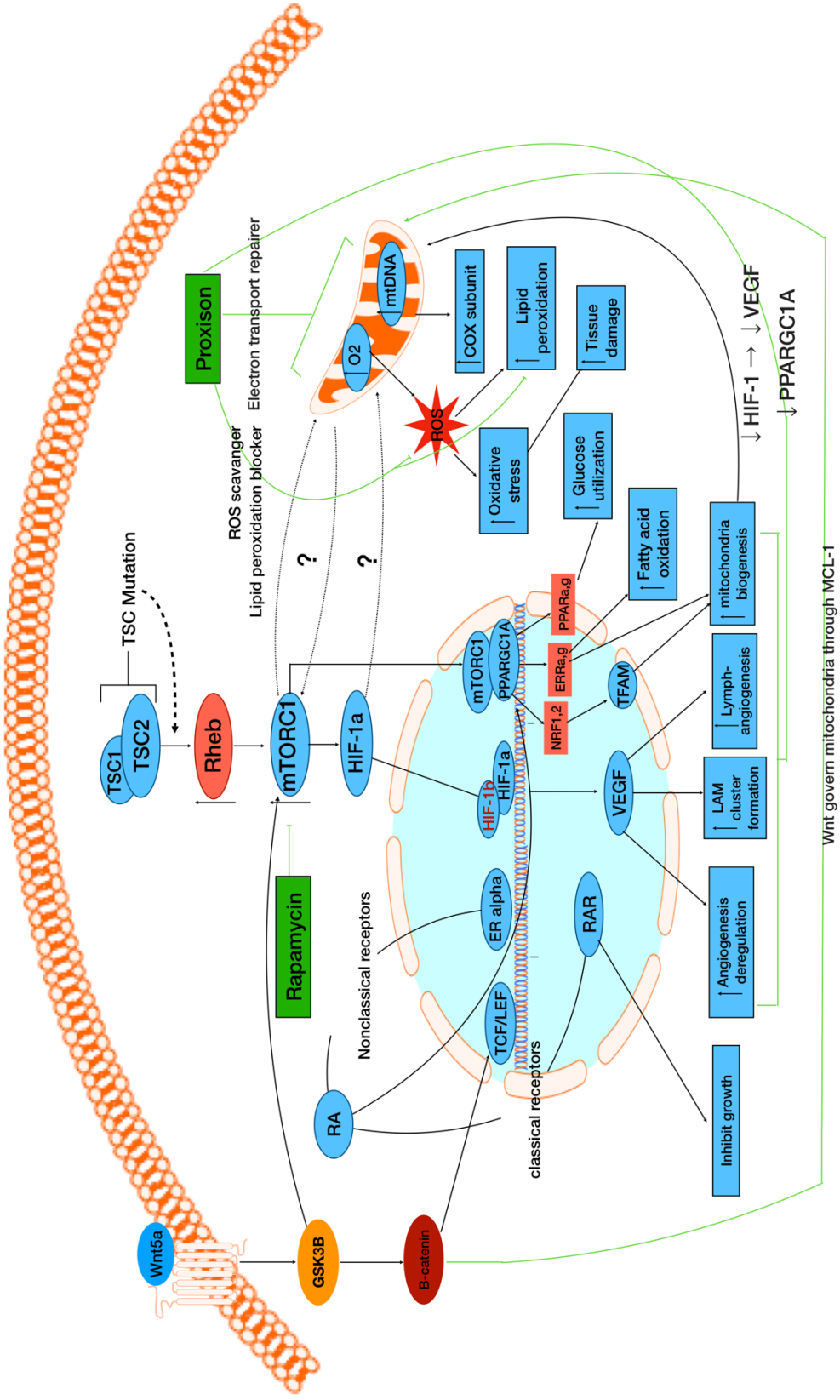

Figure 30B. Signalling pathway interactions in LAM revealing current and future therapeutic targets.

7. SUMMARY OF NOVEL FINDINGS

The study investigated the molecular background of LAM disease. The investigation led to the identification of the general deregulation of various nuclear receptors, molecules in vascularization and their miRNA regulators. The summary of these finding pointed toward mitochondrial malfunction.

Detailed study of the mitochondria using SeaHorse, Oroboros and electron microscopy confirmed mitochondrial dysfunction in LAM cells.

Proxison, a pre-clinical drug candidate with a very potent antioxidative capacity restored mitochondrial activity and corrected deregulated gene expressions implicating mitochondria as a novel target in LAM therapy.

Molecular mapping has also revealed deregulation in retinoic acid receptors. RARβ is a target for both estrogen and retinoic acid which regulate cell proliferation. Retinoic acid treatment restored normal levels of RARβ on mRNA level and also reduced proliferation rate in LAM cell lines. Data highlighted vitamin A metabolism as an additional therapeutic target in LAM.

Finally, Wnt signalling pathways have also been deregulated in LAM. Identification of specific signalling molecules might also provide further therapeutic targets for treatment of LAM sufferers.

8. FURTHER STUDIES

The present work has paved the way for the following studies:

1- Studies will be designed to assess the effect of Proxison mono and/or Proxison and Rapamycin combination therapy initially in *in vitro* LAM tissue models and primary LAM tissue cultures as well as in LAM animal models. Such studies can provide the ground work for a full clinical trial of Proxison.

2- Application of vitamin A metabolites will be assessed in LAM therapy with the aid of *in vitro* LAM tissue models and primary LAM tissue cultures.

3- Apart from vitamin A metabolism, vitamin D receptor (VDR) signalling has also been deregulated based on the low VDR expression levels. Further studies will be performed to reveal whether decreased VDR is a contributing factor to the severity of LAM.

4- The role of Wnt signalling in the regulation of LAM progression has not been investigated in detail. Based on the initial results, some molecules of the Wnt pathway ca serve as therapeutic targets. In vitro models of human LAM tissue and knock-out and knock-in molecular models can provide further insight of the role of Wnt signalling in LAM disease.

9. ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my supervisor Prof. Dr. Judit Erzsébet Pongrácz for her guidance, patience and help during this study. Without her, this work would not have been accomplished and I would have never been able to finish my PhD.

I would like to thank Prof. Dr. László Seress for his guidance and help with electron microscopy. Also, a special thank you goes to Dr. György Zsolt Miskei for his insightful advices and comments during writing of the PhD thesis plan.

I would like to thank Dr. Krisztián Kvell for his help and advices to solve the problems during my laboratory work.

I would also like to extend my thanks to all my colleagues in the Department of Pharmaceutical Biotechnology.

Finally, I would like to thank my family, especially my sister and my long term friend who always supported me during my study.

10. LIST OF PUBLICATIONS

Total impact factor: 14.541

The thesis is based on the following publications:

Published work:

Abdelwahab EMM, Pal S, Kvell K, Sarosi, V, Bai, P, Rue, R, Krymskaya, V, McPhail, D, Porter, A, Pongracz, JE: Mitochondrial dysfunction is a key determinant of the rare disease lymphangioleiomyomatosis and provides a novel therapeutic target. *Oncogene* 2018:1. doi:10.1038/s41388-018-0625-1 (IF: 6.854)

Manuscript underreviw:

Abdelwahab, ElHusseiny MM; Bovari, Judit; Smuk, Gabor; Harko, Tunde; Fillinger, Janos; Moldvay, Judit; Sarosi, Vera, Krymskaya, Vera; Pongracz, Judit E: A potential therapeutic impact of reduced retinoic acid receptor (RAR) expression in Lymphangioleiomyomatosis, 2019. British Journal of Pharmacology

Other publication:

Published work:

Abdelwahab, E.M.M., Rapp, J., Feller, D., Csongei, V., Pal, S., Bartis, D., et al. (2019). Wnt signaling regulates trans-differentiation of stem cell like type 2 alveolar epithelial cells to type 1 epithelial cells. Respir. Res. 20: 204. (IF:3.829)

Pénzes Á, **Abdelwahab EMM**, Rapp J, *et al.* Toxicology studies of primycin-sulphate using a three-dimensional (3D) in vitro human liver aggregate model. *Toxicol Lett* 2017;**281**:44–52. doi:10.1016/J.TOXLET.2017.09.005 (IF: 3.858).

Presentations:

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. Mitochondrial dysfunction is a key determinant of the rare disease Lymphangioleiomyomatosis A MAGYAR TÜDŐGYÓGYÁSZ TÁRSASÁG 2018.

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. **Mitochondrial dysfunction is a key determinant**

of the rare disease lymphangioleiomyomatosis and provides a novel therapeutic target International Cholnoky symposium 2018.

Poster:

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. **ROLE OF MITOCHONDRIA IN LYMPHANGIOLYOMATOSIS.** Targeting Mitochondria Congress Berlin 2017

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