Helmholtz-Zentrum
Geesthacht
Zentrum für Material- und Küstenforschung

Final Draft
of the original manuscript:

Felthaus, O.; Schoen, T.; Schiltz, D.; Aung, T.; Kuehlmann, B.; Jung, F.; Anker, A.; Klein, S.; Prantl, L.:

Adipose tissue-derived stem cells from affected and unaffected areas in patients with multiple symmetric lipomatosis show differential regulation of mTOR pathway genes

In: Clinical Hemorheology and Microcirculation (2018) IOS Press

DOI: 10.3233/CH-189107

Adipose Tissue-Derived Stem Cells from Affected and Unaffected Areas in Patients with Multiple Symmetric Lipomatosis show Differential Regulation of mTOR Pathway Genes

Oliver Felthaus[1,§], Teresa Schön[1], Daniel Schiltz[1], Thiha Aung[1], Britta Kühlmann[1], Friedrich Jung[2], Alexandra Anker[1], Silvan Klein[1], Lukas Prantl[1]

1: Department of Plastic -, Hand-, and Reconstructive Surgery, University Hospital Regensburg, Regensburg, Germany

2: Institute of Biomaterial Science and Berlin-Brandenburg Centre for Regenerative Therapies, Helmholtz-Zentrum Geesthacht, Kantstrasse, Teltow, Germany

Corresponding author: Dr. Oliver Felthaus, Center of Plastic -, Hand-, and Reconstructive Surgery, University Hospital Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

oliver.felthaus@ukr.de

Abstract

Background: Multiple symmetric lipomatosis is a rare disease characterized by the excessive growth of uncapsulated masses of adipose tissue. Although the etiology has yet to be elucidated, a connection to brown adipose tissue has been proposed recently. The mTOR pathway, which is found to be regulated in lipomatous tissue as well as associated with brown adipose tissue, can be inhibited by a compound called rapamycin.

Methods: We isolated adipose tissue derived stem cells from both affected and unaffected tissue and treated these cells with different concentrations of rapamycin.

Results: The differences in both proliferation and differentiation between adipose tissue derived stem cells (ASCs) from lipomatous and normal tissue decreased after mTOR pathway inhibition. In some patients regulation of mTOR genes was opposed in the ASCs from the two different tissues.

Conclusions: Treatment with rapamycin might be a novel therapeutical approach for patients suffering from multiple symmetric lipomatosis.

Key Words

Multiple Symmetric Lipomatosis, rare disease, mTOR pathway, rapamycin

35 **1. Introduction**

36 *1.1 Multiple symmetric lipomatosis*

37 The rare disease (estimated incidence 1:25,000) multiple symmetric lipomatosis (MSL; also benign
38 symmetric lipomatosis) is of unknown etiology and characterized by expansive growth of adipose
39 tissue. Patients suffering from MSL can be divided into different phenotypes. Type I (horsecollar
40 lipomata) includes exaggerated fat distribution in the neck, upper back, shoulders and upper arms;
41 type II (pseudoathletic appearance) involves exaggerated fat distribution in the shoulder girdle, deltoid
42 region, upper arms, and the thorax; type III (gynecoid distribution) is related to an excess of lipomatous
43 tissue in the lower body, especially the thighs and medial side of the knees [1]. A fourth type
44 (abdominal type) has been proposed. MSL was first described by Brodie in 1846 and further
45 characterized by Madelung in 1888 and Launois and Bensaude in 1898 [2-4]. MSL is often associated
46 with diabetes mellitus, hyperlipidaemia, hyperuricaemia, hypothyroidism, neural pathologies [5, 6]
47 and the MERRF syndrome (myoclonic epilepsy with rigged red fiber) or other mitochondrial diseases
48 [7, 8]. MSL may cause severe complications due to tracheal, laryngeal or mediastinal compression [9-
49 12]. This could lead to sleep apnea [13]. It has been reported that MSL patients with no signs of
50 coronary artery disease, acute myocardial infarction, or other cardiac abnormalities have suffered
51 from sudden cardiac death [14, 15]. The authors have linked this mortality to the occupation of the
52 mediastinal space by the lipomatous tissue. Although elevated alcohol consumption is common in
53 patients suffering from MSL, many cases are reported where patients did not drink any alcohol at all
54 [16]. Additionally, the neuropathies cannot be explained solely by the elevated alcohol consumption
55 found in most MSL patients [17, 18]. Whereas most cases are sporadic, some familial cases (inherited
56 in an autosomal-dominant fashion) have been reported [19-21]. However, the underlying genetic
57 cause is unknown. An association with brown adipose tissue (BAT) has been proposed recently [16,
58 22]. This is in accordance with the association of MSL with mitochondrial diseases, because brown fat
59 is a tissue rich in mitochondria [23, 24], and the areas generally affected by MSL mirror the distribution

of BAT [22, 25]. Additionally, adipose tissue derived stem cells (ASCs) from lipomatous tissue were found to express UCP-1, a marker for brown fat [16, 22, 26].

1.2 mTOR signaling pathway

Fat tissue homeostasis is associated with the mTOR (mechanistic target of rapamycin) signaling pathway. The pathway is important for lipid homeostasis [27] and energy homeostasis [28-30]. Additionally, a sustained activated mTOR pathway can be caused by overfeeding and can result in obesity and insulin resistance [31, 32]. Interestingly, insulin resistance is also common in patients suffering from MSL [33-35]. The mTOR gene encodes for a phosphatidylinositol kinase that, amongst others, mediates cellular responses to nutrient deprivation. Activated mTOR pathway results in the phosphorylation of activation of Ribosomal protein S6 kinase beta-1 (RPS6KB1, also p70S6 kinase, p70S6K, p70-S6K) which in turn phosphorylates S6 ribosomal protein, an activation crucial for protein synthesis. Interestingly, it was found that RPS6KB1 is important for early adipocyte differentiation [36, 37]. Using a reverse phase protein assay, we have found that genes that are regulated differentially in adipose tissue derived stem cells (ASCs) from lipomatous tissue compared to ASCs from unaffected fat tissue are overrepresented in the mTOR pathway. Among these genes were IGF, PI3K, and Akt (unpublished data).

1.3 Rapamycin

The mTOR pathway can be inhibited by rapamycin (sirolimus). Rapamycin is a compound first found in bacteria (Streptomyces hygroscopicus) from the Easter Island (Rapa Nui) [38]. Rapamycin inhibits the expression of interleukin 2 and therefore the activation of B cells and T cells and is used as an immunosuppressant after organ transplantation. Additionally, rapamycin is used as treatment for lymphangioleiomyomatosis, and stents are coated with rapamycin to suppress the proliferation of smooth muscle cells [39, 40] while the proliferation of endothelial cells is suppressed also [41].

Recently, we have shown that ASCs isolated from affected and unaffected areas can be used as a model system to evaluate differences in the regulation [24]. Additionally, we have seen that many genes regulated differentially in ASCs isolated from affected areas from MSL patients are associated with the

86 mTOR pathway, which can be inhibited by rapamycin. Here, we show that ASCs from normal and
87 lipomatous tissue react differently to rapamycin treatment and that genes associated with the mTOR-
88 pathway are regulated differentially in these ASCs.

89

90 ## 2. Methods

91 *2.1 Cell culture*

92 Cells from fat tissue of affected and unaffected areas from five patients were isolated as previously
93 described [24, 42]. Briefly, surgical biopsies from lipomatous tissue and normal adipose tissue were
94 obtained. All patients were given verbal and written information, and signed informed consent was
95 obtained prior to study start. The study was approved by the Independent Ethic Committee of the
96 University of Regensburg (No.: 08/117). Subcutaneous adipose tissue (10g±0.5g) was harvested under
97 local superficial skin anesthesia with Xylocaine. Tissue sample was used for extraction of adipose tissue
98 derived stem cells (ASCs). The fat tissue was minced into small pieces (1mm^3) and subsequently
99 digested with collagenase (Sigma-Aldrich, St. Louis, MO, USA; 5U/ml fat tissue) for 45 minutes. The
100 solution was filtered through a 100-µm strainer, centrifuged (500 rcf) and the cells from the obtained
101 pellet were seeded into culture flasks with MEM alpha1 medium (Sigma Aldrich) containing 20% heat-
102 inactivated FBS (Pan-Biotech, Aidenbach, Germany), 2 mM glutamine (Thermo Fisher, Waltham, MA,
103 USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) and were incubated at 37°C in
104 a humidified atmosphere containing 5% CO_2. Upon reaching subconfluency, cells were detached with
105 a 0.25% trypsin/0.02% EDTA solution (Pan-Biotech) and seeded at a density of 5000 cells/cm^2. Cells in
106 passage 5 were used for all experiments. For adipogenic differentiation, the cell culture medium was
107 additionally supplemented with 1 µM dexamethasone, 0.5 mM IBMX, 100 µM indomethacin, and 10
108 µg/ml human recombinant insulin (all Sigma-Aldrich). For inhibition of the mTOR signaling pathway
109 the medium was supplemented with rapamycin (Sigma Aldrich, 0 ng/ml, 1 ng/ml, or 10 ng/ml,
110 respectively). After a time period indicated in the respective result section, the cells were either used
111 for vitality assays, stained for detection of adipogenic differentiation, or harvested for RNA isolation.

112 *2.2 Cell viability assay*

113 Cell viability was assessed using resazurin (Sigma-Aldrich) which is metabolized to the fluorescent
114 resorufin by vital cells. ASCs were seeded into 96-well plates and allowed to adhere for 24h.
115 Subsequently, the proliferation medium was discarded and replaced by medium supplemented with
116 different rapamycin concentrations. After the time indicated in the respective experiments, the
117 medium was replaced with a 0.07 mM resazurin in proliferation medium-solution. After 2 hours
118 fluorescence intensity (excitation 530 nm, emission 590 nm) was measured using the VarioScan plate
119 reader (Thermo Fisher). Results are shown as mean of all five patients ± SD.

120 *2.3 Oil red O staining*

121 Staining with Oil red O was used to assess adipogenic differentiation. Cells were cultured until
122 subconfluency was reached. Subsequently, the medium was changed to adipogenic differentiation
123 medium. After ten days, cells were fixed with a 10% formalin (Sigma Aldrich) solution for 10 minutes.
124 After washing with PBS, a 5 mM oil red O in 60% isopropanol solution was added and the cells were
125 stained for 20 minutes. For quantification, the dye was discarded and the cells were washed with PBS
126 four times and with 60% isopropanol one time. Subsequently, oil red O was eluted from the cellular
127 lipid droplets with 100% isopropanol. Optical density at 518 nm wavelength was measured using the
128 VarioScan plate reader. Results are shown as mean of all five patients ± SD.

129 *2.4 Real-Time RT-PCR*

130 Cells were seeded into 6-well plates and upon subconfluency treated with the different rapamycin
131 concentrations for seven days. For RNA isolation, the RNeasy mini Kit (Qiagen, Hilden, Germany) was
132 used according to the manufacturer's instructions. RNA concentration was measured using a
133 NanoDrop 2000 Spectrophotometer (Thermo Fisher) and reverse transcription of 1 µg total RNA was
134 done using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was done using the
135 DyNaMo Color Flash SYBR Green Master Mix (Thermo Fisher) and the Eco Thermal cycler (Illumina, San
136 Diego, CA, USA). Primer sequences are as follows: GAPDH_forward: 5`-GAAAGATGGTGATGGGATTTC-
137 3`, GAPDH_reverse: 5`-GAAGGTGAAGGTCGGAGTC-3`; mTOR_forward: 5`-CGAAGCCGCGCGAACC-3`,

mTOR_reverse: 5`-ATTCCGGCTCTTTAGGCCAC-3`; EIF4EBP1_forward: 5`-GGAGTGTCGGAACTCACCTG-3`, EIF4EBP1_reverse: 5`-ACTGTGACTCTTCACCGCC-3`; RPS6K1_forward: 5`-TGTCGACAGCCCAGATGACT-3`, RPS6K1_reverse: 5`-ATTTGACTGGGCTGACAGGT-3`. Experiments were done in triplicates. The $\Delta\Delta_{Ct}$-method was used to calculate relative gene expression with GAPDH as housekeeping gene. Results are shown as mean ± SD for every patient. Relative gene expression is normalized to ASCs.

2.5 Statistics

Statistics was done using Student's T-Test. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

3. Results

3.1 Cell viability assay

ASCs from lipomatous tissue showed higher cell viability when compared to ASCs from normal tissue. Treatment with rapamycin for 48 hours led to a decrease in cell viability in both 1 and 10 ng/ml concentrations in the ASCs from both normal and lipomatous tissue. After treatment with rapamycin in both concentrations the cells from the unaffected areas had around 65% of their original viability remained. In contrast, cell viability of ASCs from affected tissue decreased to around 35% of their former value (figure 1).

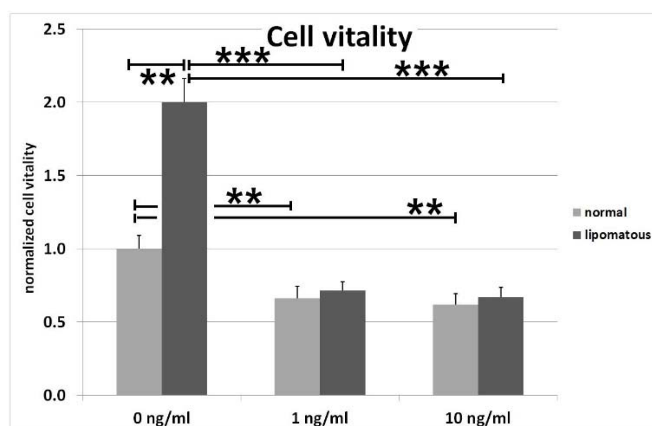
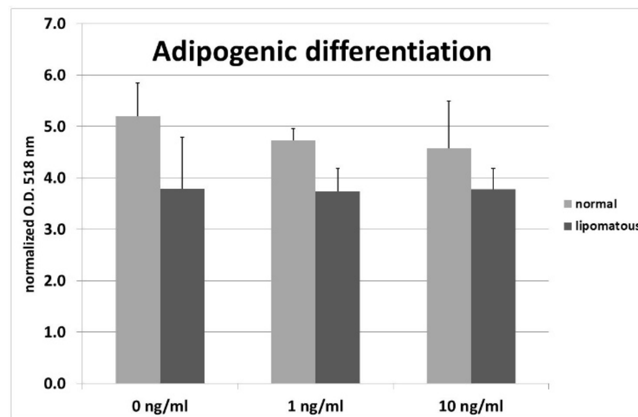


Figure 1: Normalized cell vitality after 48 hours of ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Results are shown as mean of all five patients ± SD. Significance is indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

159

160 *3.2 Oil red O staining*

161 ASCs from normal tissue showed a higher degree of differentiation when compared to ASCs from
162 lipomatous tissue. Adipogenic differentiation was impaired in ASCs from normal tissue when
163 rapamycin was added to the culture medium. After treatment with 1 ng/ml and 10 ng/ml rapamycin
164 Oil Red O staining decreased to 91% or 88% of the original values, respectively. Adipogenic
165 differentiation of ASCs from lipomatous tissue was nearly unaffected (Figure 2).



166

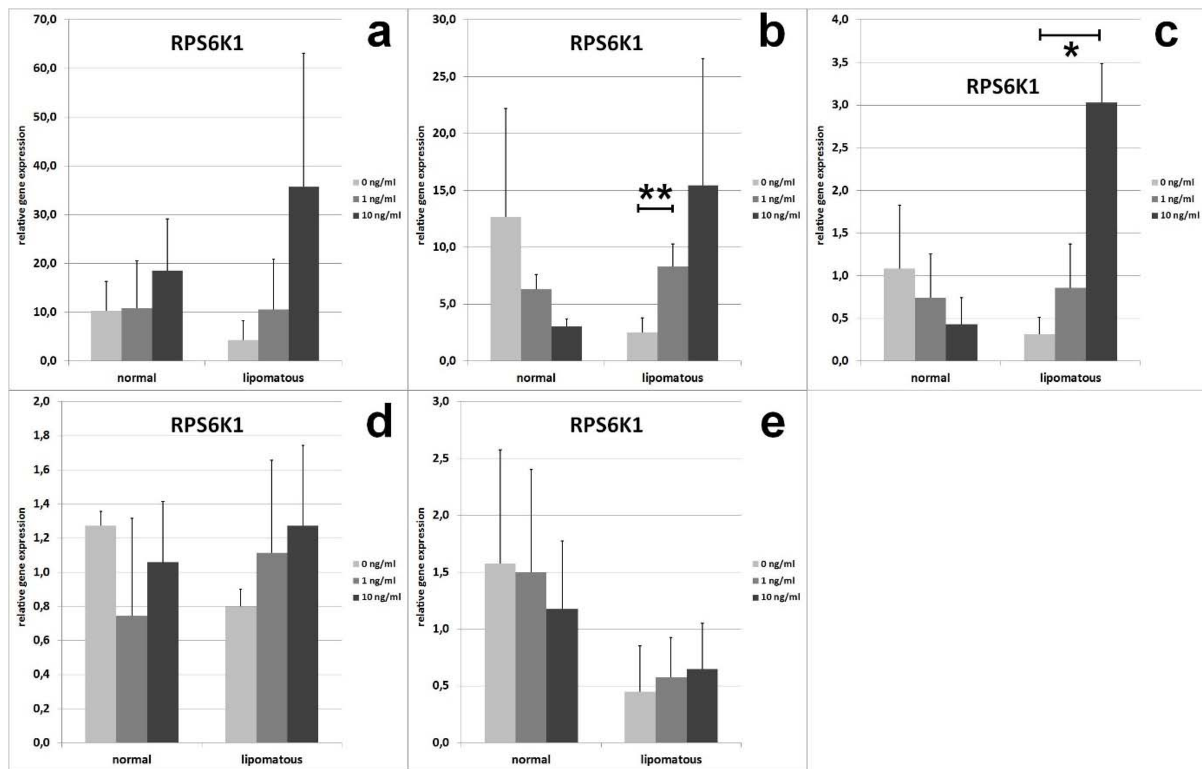
167 Figure 2: Adipogenic differentiation of ASCs from normal and lipomatous tissue after treatment with 0 ng/ml, 1
168 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were differentiated for 10 days and stained with Oil Red O
169 subsequently. After washing, the dye bound to triglycerides was eluted with isopropanol and measured
170 colorimetrically. Values are normalized to undifferentiated ASCs from normal tissue. Results are shown as mean
171 of all five patients ± SD. No statistically significant differences were observed.

172

173 *3.3 Real-Time (RT)-PCR*

174 Gene expression was regulated differentially in ASCs from affected and unaffected areas after
175 rapamycin exposition in some patients. This was most considerable for the direct target of the mTOR
176 signaling pathway, RPS6K1, but also seen in some patients for EIF4EBP1 and mTOR. RPS6K1 was
177 downregulated with increasing rapamycin concentrations in ASCs from normal tissue but upregulated
178 in ASCs from lipomatous tissue (Figures 3b-e). Patient 1, however, did not show this opposed
179 regulation (Figure 3a). EIF4EBP1 was downregulated or not regulated with increasing rapamycin
180 concentrations in ASCs from unaffected tissue but upregulated in ASCs from affected tissue in some

181 patients (Figures 4a, d). Patients 2, 3, and 5, however, did not show this opposed regulation (Figures
182 4b, c, e). mTOR was downregulated with increasing rapamycin concentrations in ASCs from normal
183 tissue but upregulated in ASCs from lipomatous tissue (Figures 5b, c). Patients 1, 4, and 5 however, did
184 not show this opposed regulation (Figures 5a, d, e).



185

186 Figure 3: Relative gene expression of RPS6K1 in ASCs from normal and lipomatous tissue after treatment with 0
187 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized to
188 undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is
189 indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

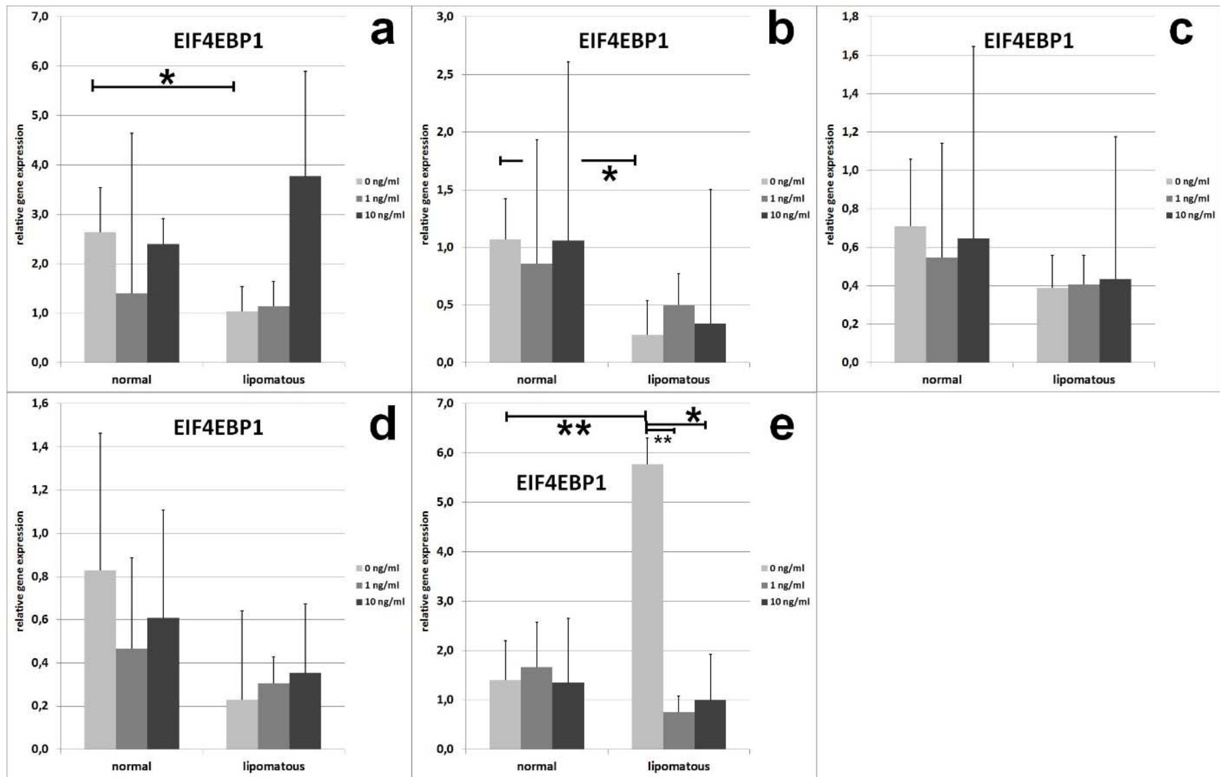
190

191 **4. Discussion**

192 Surgical removal of lipomatous tissue via lipectomy or liposuction provides to date the only validated
193 therapeutic approach for patients suffering from MSL [16]. However, relapse after surgery is occurring
194 frequently [43, 44]. Although the term benign symmetrical lipomatosis implies a mere
195 aesthetic/cosmetic problem, the patients not only suffer from severe psychological strain but also from
196 accessory symptoms that can range from decreased quality of life (tracheal, laryngeal or mediastinal
197 compression, sleep apnea) to sudden cardiac death. Because of this, the justification of the term

198 benign in benign symmetrical lipomatosis has been doubted [14]. Therefore, a pharmaceutical therapy
199 would be beneficial. In the recent past increasing evidence that BAT might be involved in the etiology
200 of MSL has been reported. The mTOR pathway is associated with lipid homeostasis in general and in
201 particular with the regulation of BAT. Additionally, we have found several genes associated with the
202 mTOR pathway to be regulated differentially in ASCs from lipomatous tissue. Therefore, we have
203 conducted a study to evaluate the influence of the mTOR pathway inhibiting compound rapamycin on
204 ASCs isolated from unaffected and affected tissues from patients suffering from MSL. It was shown
205 that ASCs from lipomatous tissue have a higher proliferation rate but an impaired differentiation
206 capacity when compared with normal tissue ASCs. This is in accordance with the conclusion, that MSL
207 is a hyper proliferative stem cells disorder [26]. After treatment with rapamycin, the cells from the two
208 different tissue origins aligned their proliferation and differentiation behavior. Cell viability was much
209 more impaired in ASCs from lipomatous tissue than in those from unaffected areas. As a result, both
210 cell types had a similar viability (Figure 1). Differentiation on the other hand was impaired significantly
211 in normal tissue ASCs but not in ASCs from lipomatous tissue, making both types becoming more alike
212 (Figure 2). Interestingly, this might at least in part be due to the opposite regulation of mTOR pathway
213 related genes in these ASCs. Real Time RT-PCRs have shown that in some patients, the two different
214 cell types showed a contrary regulation of mTOR, EIF4EBP1, and RPS6K1 when treated with rapamycin.
215 In some patients, after rapamycin incubation these genes are downregulated or not regulated in ASCs
216 from normal tissue but upregulated in ASCs from lipomatous tissue (Figures 3-5).

217

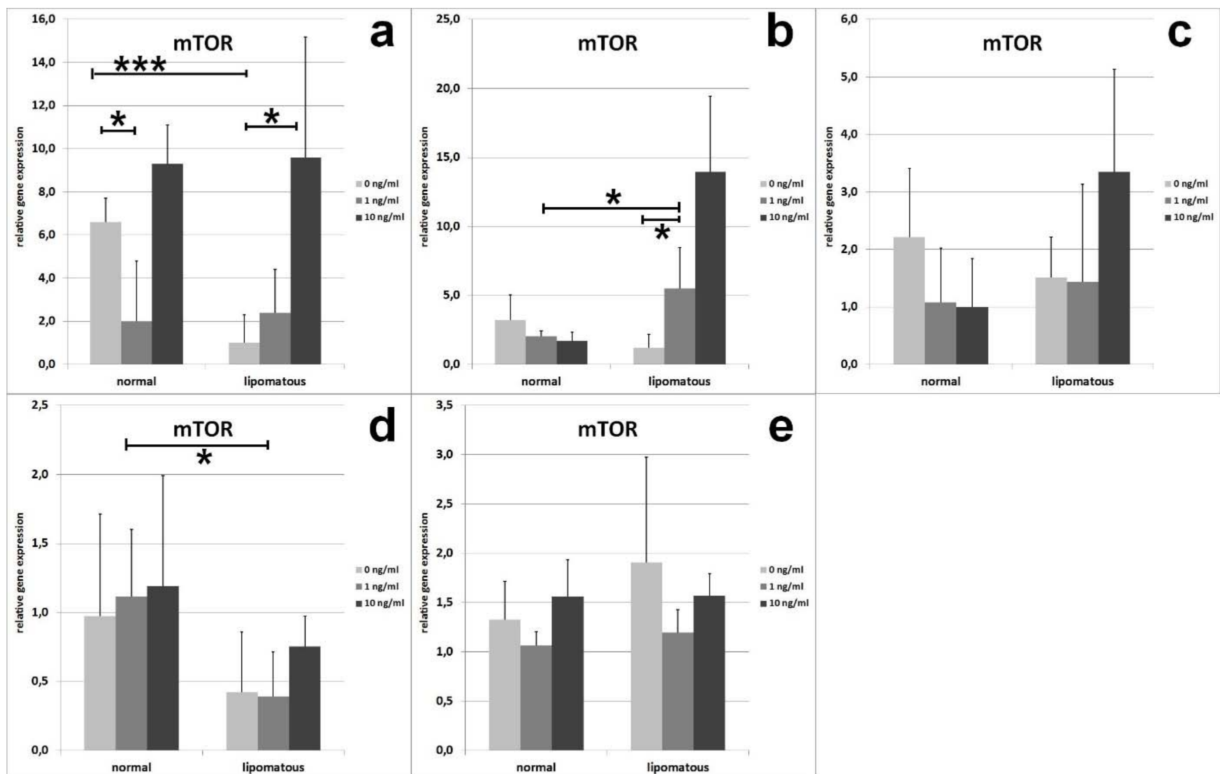


218 Figure 4: Relative gene expression of EIF4EBP1 in ASCs from normal and lipomatous tissue after treatment with

219 0 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized

220 to undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is

221 indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.



222

223 Figure 5: Relative gene expression of mTOR in ASCs from normal and lipomatous tissue after treatment with 0
224 ng/ml, 1 ng/ml, and 10 ng/ml rapamycin, respectively. Cells were incubated for 7 days. Values are normalized to
225 undifferentiated ASCs from normal tissue. Results are shown as mean for every patient ± SD. Significance is
226 indicated as follows: *: p-value < 0.05; **: p-value < 0.005; ***: p-value < 0.001.

227

228 However, this regulation was not observed in ASCs from all patients. On the other hand, nearly no
229 accessory symptoms and causes that have been proposed for MSL can be applied to all patients. This
230 has already been reported for alcohol abuse, neuropathies, and for mitochondrial genome mutations,
231 which can be seen in many but not in all patients suffering from MSL [45, 46]. Although additional
232 research is needed to address this question, we can present some evidence to support the hypothesis
233 that mTOR is involved in the pathogenesis of MSL. First of all, mTOR plays a crucial role in lipid
234 homeostasis and adipocyte maturing [47], which might be impaired in ASCs from affected tissues.
235 Moreover, mTOR pathway is involved in BAT regulation, from which lipomatous tissue has been
236 proposed to origin [16, 22, 25, 26]. Lbk1, a gene known to control BAT expansion and UCP-1 expression
237 in mice, exerts is function partly via mTOR signaling pathway [48]. Deletion of mTOR induced white
238 adipose tissue to adopt characteristics from BAT [49]. However, negative impacts of rapamycin on UCP-
239 1 expression in BAT have been reported, too [50]. Associated with the mTOR pathway is Sestrin2, a
240 regulator of thermogenesis in BAT [51]. Moreover, in a previous study we have seen that the proteins
241 found regulated differentially in ASCs isolated from affected areas are overrepresented in the mTOR
242 signaling pathway (IGF, PI3K, Akt). Interestingly, an association of MSL with the mTOR pathway related
243 gene PTEN has been proposed earlier [52-54]. Another connection between mTOR pathway and MSL
244 is insulin resistance, which is common in patients and can result from both overfeeding and a sustained
245 active mTOR pathway [31-35]. Interestingly, both caloric restriction and rapamycin have a positive
246 effect on murine lifespan [55, 56]. Whether the longer life expectance of mice treated with rapamycin
247 is comparable with the life-extending effects of dietary restriction is yet to be elucidated. However,
248 humans cannot be compared with rodents regarding the positive effects on lifespan of caloric
249 restriction [55, 57]. Rapamycin is already used for several indications. The drug is marketed under the

250 trade names Rapamune® (Pfizer) or Certican® (Novartis), respectively. Rapamycin, in a medical
251 background often called sirolimus, is used as an immunosuppressant to prevent rejection after kidney,
252 liver, or heart transplantation and is also used as a treatment for renal cell carcinoma [58-62].
253 Additionally, Sirolimus is used for coronary stent coating [39]. Additionally to these indications, orphan
254 designation was granted by the European Commission for sirolimus for the treatment of chronic non-
255 infectious uveitis (EU/3/11/898, Santen Oy, Finland, 30 August 2011), for the prevention of
256 arteriovenous access dysfunction in patients undergoing surgical creation of an arteriovenous access
257 for hemodialysis (EU/3/13/1204, S-Cubed Limited, United Kingdom, 13 November 2013), for the
258 treatment of tuberous sclerosis (EU/3/15/1557, Desitin Arzneimittel GmbH, Germany, 9 October
259 2015), and for the treatment of beta-thalassaemia intermedia and major (EU/3/15/1585, Rare Partners
260 srl Impresa Sociale, Italy, 14 December 2015). Here, we show a possible novel therapeutic approach
261 for future therapies of patients suffering from MSL.
262

263 ## 5. Conclusion

264 The mTOR pathway might be involved in the pathogenesis of MSL. Rapamycin could be an approach
265 to a novel pharmaceutical therapy. As already reported for alcohol abuse and for mitochondrial
266 genome mutations [45, 46], this is not necessarily true for all patients.
267

268 *5.1 Competing interests*

269 We declare that there are no competing interests.
270

271 *Acknowledgements*

272 This work was supported by the Helmholtz-Association through programme-oriented funding. We wish
273 to thank Bernadette Fürnstein for her contribution in cell culture.
274 This paper is dedicated to the 70th birthday of Prof. Friedrich Jung.
275

References

[1] Donhauser G, Vieluf D, Ruzicka T, Braun-Falco O. Benign symmetric Launois-Bensaude type III lipomatosis and Bureau-Barrière syndrome. *Hautarzt*. 1991;42:311–314.

[2] Brodie, B. Lectures illustrative of various subjects in pathology and surgery. In, Longman, London, 1846, pp. 275-276.

[3] Madelung, O. Über den Fetthals. *Langenbecks Archiv Klin Chirurg*. 1888;37,106.

[4] Launois PE, Bensaude R. De l'adéno lipomatose symétrique. *Bull Soc Méd Hôp Paris*, Mémoires 1898;1:298–318

[5] Gdynia HJ, Sperfeld AD, Knirsch U, Homberg V, Rosenbohm A, Müller-Höcker J, Ludolph AC, Hanemann CO. Benign symmetric lipomatosis with axonal neuropathy and abnormalities in specific mitochondrial tRNA regions. *Eur J Med Res*. 2006;11(12):545-6.

[6] Sawyer SL, Cheuk-Him Ng A, Innes AM, Wagner JD, Dyment DA, Tetreault M; Majewski J, Boycott KM, Screaton RA, Nicholson G. Homozygous mutations in MFN2 cause multiple symmetric lipomatosis associated with neuropathy. *Hum Mol Genet*. 2015;24(18):5109-14.

[7] Holme E, Larsson NG, Oldfors A, Tulinius M, Sahlin P, Stenman G. Multiple symmetric lipomas with high levels of mtDNA with the tRNA(Lys) A-->G(8344) mutation as the only manifestation of disease in a carrier of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome. *Am J Hum Genet*. 1993;52(3):551-6.

[8] Gámez J, Playán A, Andreu AL, Bruno C, Navarro C, Cervera C, Arbós MA, Schwartz S, Enriquez JA, Montoya J. Familial multiple symmetric lipomatosis associated with the A8344G mutation of mitochondrial DNA. *Neurology*. 1998;51(1):258-60.

[9] Enzi G, Digito M, Marin R, Carraro R, Baritussio A, Manzato E: Mediastino-abdominal lipomatosis: Deep accumulation of fat mimicking a respiratory disease and ascites. Clinical aspects and metabolic studies *in vitro*. *Q J Med* 1984,53:453-463.

301 [10] Senturk T, Akar H, Kadikoilu G. Multiple symmetric lipomatosis with mediastinal involvement
302 and subclinical hypothyroidism in a female patient. *T Klin Med Sci.* 2002;22:413e6.
303 [11] Ramos S, Pinheiro S, Diogo C, Cabral L, Cruzeiro C: Madelung disease: A not-so-rare disorder.
304 *Ann Plast Surg*. 2010,64:122-124.
305 [12] Milisavljevic D, Zivic M, Stankovic P. Severe dyspnea as atypical presenting symptom of
306 madelung's disease. *Hippokratia*. 2010;14:133e5.
307 [13] Fonseca VA, Alves C, Marques H, Camacho E, Saraiva AP. Madelung's disease as a rare cause of
308 obstructive sleep apnea. *J Bras Pneumol*. 2009;35(10):1053-6.
309 [14] Enzi G, Busetto L, Ceschin E, Coin A, Digito M, Pigozzo S. Multiple symmetric lipomatosis: Clinical
310 aspects and outcome in a long-term longitudinal study. *Int J Obes Relat Metab Disord*.
311 2002;26(2):253-61.
312 [15] Fonseca VR, Freitas C, Palmeira M, Ferreira C, Victorino R. Cardiac noradrenergic denervation in
313 a patient with multiple symmetric lipomatosis. *Cardiology*. 2012;121(3):160-3.
314 [16] Enzi G, Busetto L, Sergi G, Coin A, Inelmen EM, Vindigni V, Bassetto F, Cinti S. Multiple symmetric
315 lipomatosis: A rare disease and its possible links to brown adipose tissue. *Nutr Metab Cardiovasc*
316 *Dis.* 2015;25(4):347-53.
317 [17] Naumann M, Schalke B, Klopstock T, Reichmann H, Lange KW, Wiesbeck G, Toyka KV, Reiners K.
318 Neurological multisystem manifestation in multiple symmetric lipomatosis: A clinical and
319 electrophysiological study. *Muscle Nerve*. 1995;18(7):693-8.
320 [18] Klopstock T, Naumann M, Schalke B, Bischof F, Seibel P, Kottlors M, Eckert P, Reiners K, Toyka
321 KV, Reichmann H. Multiple symmetric lipomatosis: Abnormalities in complex IV and multiple
322 deletions in mitochondrial DNA. *Neurology*. 1994;44(5):862-6.
323 [19] Meningaud JP1, Pitak-Arnnop P, Bertrand JC. Multiple symmetric lipomatosis: Case report and
324 review of the literature. *J Oral Maxillofac Surg*. 2007;65(7):1365-9.
325 [20] Ardeleanu V, Chicos S, Georgescu C, Tutunaru D. Multiple benign symmetric lipomatosis -- a
326 differential diagnosis of obesity. *Chirurgia (Bucur).* 2013;108(4):580-3.

327 [21] Chalk CH, Mills KR, Jacobs JM, Donaghy M. Familial multiple symmetric lipomatosis with
328 peripheral neuropathy. *Neurology*. 1990;40(8):1246-50.
329 [22] Plummer C1, Spring PJ, Marotta R, Chin J, Taylor G, Sharpe D, Athanasou NA, Thyagarajan D,
330 Berkovic SF. Multiple Symmetrical Lipomatosis--a mitochondrial disorder of brown fat.
331 *Mitochondrion*. 2013;13(4):269-76.
332 [23] Schulz TJ, Tseng Y. Brown adipose tissue: Development, metabolism and beyond. Biochem J.
333 2013; 453(2): 10.1042/BJ20130457.
334 [24] Cedikova M, Kripnerová M, Dvorakova J, Pitule P, Grundmanova M, Babuska V, Mullerova D,
335 Jitka Kuncova J. Mitochondria in White, Brown, and Beige Adipocytes. Stem Cells Int. 2016;
336 2016: 6067349.
337 [25] Kodish ME, Alsever RN, Block MB. Benign symmetric lipomatosis: Functional sympathetic
338 denervation of adipose tissue and possible hypertrophy of brown fat. *Metabolism*.
339 1974;23(10):937-45.
340 [26] Prantl L, Schreml J, Gehmert S, Klein S, Bai X, Zeitler K, Schreml S, Alt E, Gehmert S, Felthaus O.
341 Transcription Profile in Sporadic Multiple Symmetric Lipomatosis Reveals Differential Expression
342 at the Level of Adipose Tissue-Derived Stem Cells. *Plast Reconstr Surg*. 2016;137(4):1181-90.
343 [27] Lamming DW, Sabatini DM. A Central role for mTOR in lipid homeostasis. Cell Metab.
344 2013;18(4):465-9.
345 [28] Mannaa M, Krämer S, Boschmann M, Gollasch M. mTOR and regulation of energy homeostasis
346 in humans. *J Mol Med (Berl).* 2013;91(10):1167-75.
347 [29] Albert V, Hall MN2. mTOR signaling in cellular and organismal energetics. *Curr Opin Cell Biol*.
348 2015;33:55-66.
349 [30] Yang A, Xiao X, Zhao M, LaRue AC, Schulte BA, Wang GY. Differential Reponses of
350 Hematopoietic Stem and Progenitor Cells to mTOR Inhibition. Stem Cells Int.
351 2015;2015:561404.

352 [31] Dann SG, Selvaraj A, Thomas G. mTOR Complex1-S6K1 signaling: At the crossroads of obesity,
353 diabetes and cancer. *Trends Mol Med*. 2007;13(6):252-9.
354 [32] Małgorzewicz S, Dardzińska JA, Gnacińska M, Jankun J, Bryl E, Sworczak K. Complex interaction
355 between the immune system and adipose tissue (Review). *Int J Mol Med*. 2014;33(1):3-7.
356 [33] Harsch IA, Schahin SP, Fuchs FS, Hahn EG, Lohmann T, Konturek PC, Ficker JH. Insulin resistance,
357 hyperleptinemia, and obstructive sleep apnea in Launois-Bensaude syndrome. *Obes Res*.
358 2002;10(7):625-32.
359 [34] Cojocaru DC, Cozma CD, Postolache P. Markers of insulin resistance in a case of Launois-
360 Bensaude syndrome. *Rev Med Chir Soc Med Nat Iasi*. 2013;117(2):404-8.
361 [35] D'Ettorre M, Gniuli D, Guidone C, Bracaglia R, Tambasco D, Mingrone G. Insulin sensitivity in
362 Familial Multiple Lipomatosis. *Eur Rev Med Pharmacol Sci*. 2013;17(16):2254-6.
363 [36] Carnevalli LS, Masuda K, Frigerio F, Le Bacquer O, Um SH, Gandin V, Topisirovic I, Sonenberg N,
364 Thomas G, Kozma SC. S6K1 plays a critical role in early adipocyte differentiation. *Dev Cell*.
365 2010;18(5):763-74.
366 [37] Wu X, Schneider N, Platen A, Mitra I, Blazek M, Zengerle R, Schüle R, Meier M. In situ
367 characterization of the mTORC1 during adipogenesis of human adult stem cells on chip. *Proc*
368 *Natl Acad Sci U S A.* 2016;113(29):E4143-50.
369 [38] Vézina C, Kudelski A, Sehgal SN. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy
370 of the producing streptomycete and isolation of the active principle. *J Antibiot (Tokyo).*
371 1975;28(10):721-6.
372 [39] Abizaid A. Sirolimus-eluting coronary stents: A review. *Vasc Health Risk Manag*. 2007;3(2):191-
373 201.
374 [40] Anadol R, Gori T. The mechanisms of late scaffold thrombosis. Clin Hemorheol Microcirc.
375 2017,67(3-4): 343-346.

376 [41] Krüger-Genge A, Hiebl B, Franke RP, Lendlein A, Jung F. Effects of Tacrolimus or Sirolimus on the
377 adhesion of vascular wall cells: Controlled *in-vitro* comparison study. Clin Hemorheol Microcirc.
378 2017;67(3-4): 309-318.
379 [42] Wankhade UD, Shen M, Kolhe R, Sadanand Fulzele S. Advances in Adipose-Derived Stem Cells
380 Isolation, Characterization, and Application in Regenerative Tissue Engineering. Stem Cells Int.
381 2016; 2016: 3206807.
382 [43] Hadjiev B, Stefanova P, Shipkov C, Uchikov A, Mojallal A. Madelung disease: On the morphologic
383 criteria for diagnosis and treatment. *Ann Plast Surg*. 2010;64(6):807-8.
384 [44] Brea-García B, Cameselle-Teijeiro J, Couto-González I, Taboada-Suárez A, González-Álvarez E.
385 Madelung's disease: Comorbidities, fatty mass distribution, and response to treatment of 22
386 patients. *Aesthetic Plast Surg*. 2013;37(2):409-16.
387 [45] Heckmann JG, Kraus B, Stefan H, Neundörfer B. Progressive muscle pain, weight gain, fatique
388 and unusual body shape change. *Eur J Neurol*. 2000;7(2):243-4.
389 [46] Klopstock T, Naumann M, Seibel P, Shalke B, Reiners K, Reichmann H. Mitochondrial DNA
390 mutations in multiple symmetric lipomatosis. *Mol Cell Biochem*. 1997;174(1-2):271-5.
391 [47] Blanchard PG, Festuccia WT, Houde VP, St-Pierre P, Brûlé S, Turcotte V, Côté M, Bellmann K,
392 Marette A, Deshaies Y. Major involvement of mTOR in the PPARγ-induced stimulation of adipose
393 tissue lipid uptake and fat accretion. *J Lipid Res*. 2012;53(6):1117-25.
394 [48] Shan T, Xiong Y, Zhang P, Li Z, Jiang Q, Bi P, Yue F, Yang G, Wang Y, Liu X, Kuang S. Lkb1 controls
395 brown adipose tissue growth and thermogenesis by regulating the intracellular localization of
396 CRTC3. *Nat Commun*. 2016;7:12205.
397 [49] Shan T, Zhang P, Jiang Q, Xiong Y, Wang Y, Kuang S. Adipocyte-specific deletion of mTOR inhibits
398 adipose tissue development and causes insulin resistance in mice. *Diabetologia*.
399 2016;59(9):1995-2004.
400 [50] García-Casarrubios E, de Moura C, Arroba AI, Pescador N, Calderon-Dominguez M, Garcia L,
401 Herrero L, Serra D, Cadenas S, Reis F, Carvalho E, Obregon MJ, Valverde ÁM. Rapamycin

402 negatively impacts insulin signaling, glucose uptake and uncoupling protein-1 in brown
403 adipocytes. *Biochim Biophys Acta*. 2016;1861(12 Pt A):1929-1941.
404 [51] Ro SH, Semple I, Ho A, Park HW, Lee JH. Sestrin2, a Regulator of Thermogenesis and
405 Mitohormesis in Brown Adipose Tissue. *Front Endocrinol (Lausanne*). 2015;6:114.
406 [52] Muñoz-Málaga A, Bautista J, Salazar JA, Aguilera I, Garcia R, Chinchon I, Segura MD, Campos Y,
407 Arenas J: Lipomatosis, proximal myopathy, and the mitochondrial 8344 mutation. A lipid storage
408 myopathy? *Muscle Nerve.* 2000,23:538-542.
409 [53] Berkovic SF, Andermann F, Shoubridge EA, Carpenter S, Robitaille Y, Andermann E, Melmed C,
410 Karpati G: Mitochondrial dysfunction in multiple symmetrical lipomatosis. *Ann Neurol*.
411 1991,29:566-569.
412 [54] Kratz C, Lenard HG, Ruzicka T, Gärtner J: Multiple symmetric lipomatosis: An unusual cause of
413 childhood obesity and mental retardation. *Eur J Paediatr Neurol.* 2000,4:63-67.
414 [55] Liao CY, Rikke BA, Johnson TE, Gelfond JA, Diaz V, Nelson JF. Fat maintenance is a predictor of
415 the murine lifespan response to dietary restriction. *Aging Cell.* 2011;10(4):629-39.
416 [56] Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, Nadon NL, Wilkinson JE, Frenkel
417 K, Carter CS, Pahor M, Javors MA, Fernandez E, Miller RA. Rapamycin fed late in life extends
418 lifespan in genetically heterogeneous mice. *Nature*. 2009;460(7253):392-5.
419 [57] Zhao G, Guo S, Somel M, Khaitovich P. Evolution of human longevity uncoupled from caloric
420 restriction mechanisms. *PLoS One.* 2014;9(1):e84117.
421 [58] Deyo JC, Nicolsen N, Lachiewicz A, Kozlowski T. Salvage Treatment of Mucormycosis Post-Liver
422 Transplant With Posaconazole During Sirolimus Maintenance Immunosuppression. *J Pharm*
423 *Pract.* 2016. pii: 0897190016628702.
424 [59] Karakas NM, Erdogan I, Ozdemir B, Sezgin A. Pain Syndrome and Ventricular Arrhythmia Induced
425 by Sirolimus and Resolved by Dosage Adjustment in a Child After Heart Transplant: A Case
426 Report. *Exp Clin Transplant.* 2016, doi: 10.6002/ect.2015.0320.

427 [60] Haasova M, Snowsill T, Jones-Hughes T, Crathorne L, Cooper C, Varley-Campbell J, Mujica-Mota
428 R, Coelho H, Huxley N, Lowe J, Dudley J, Marks S, Hyde C, Bond M, Anderson R.
429 Immunosuppressive therapy for kidney transplantation in children and adolescents: Systematic
430 review and economic evaluation. *Health Technol Assess*. 2016;20(61):1-324.
431 [61] Nguyen QD, Merrill PT, Clark WL, Banker AS, Fardeau C, Franco P, LeHoang P, Ohno S, Rathinam
432 SR, Thurau S, Abraham A, Wilson L, Yang Y, Shams N. Intravitreal Sirolimus for Noninfectious
433 Uveitis: A Phase III Sirolimus Study Assessing Double-masked Uveitis Treatment (SAKURA).
434 *Ophthalmology. 2016;123(11):2413-2423.*
435 [62] Ta MH, Schwensen KG, Foster S, Korgaonkar M, Ozimek-Kulik JE, Phillips JK, Peduto A, Rangan
436 GK. Effects of TORC1 Inhibition during the Early and Established Phases of Polycystic Kidney
437 Disease. *PLoS One*. 2016;11(10):e0164193.