bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1
2
3
4
5

Modulation of ADARs mRNA expression in congenital heart defect patients

Faiza Altaf[1,#], Cornelia Vesely[3,¶], Abdul Malik Sheikh[2,&], Rubab Munir[2,&], Syed Tahir Abbass Shah[1,#,*], Aamira Tariq[1,#,*]

[1#.]Department of Biosciences , Comsats Institute of Information Technology , Islamabad, Pakistan

[2,&.]Rawalpindi Institute of Cardiology, Rawalpindi, Pakistan

[3,.¶]Center for Anatomy and Cell Biology, Medical University Vienna, Schwarzspanierstrasse 17-I, 1090 Vienna, Austria.

*Correspondence aamira_tariq@comsats.edu.pk, syedtahirabbas@comsats.edu.pk

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

27 **Abstract**

28 Adenosine (A) to inosine (I) RNA editing, is a hydrolytic deamination reaction catalyzed by
29 adenosine deaminase (ADAR) acting on RNA enzymes. RNA editing is a molecular process that
30 involves the post-transcriptional modification of RNA transcripts. Interestingly, few studies have
31 been carried out to determine the role of RNA editing in vascular disease. The current study
32 found that in blood samples positive for congenital heart disease (CHD) ADAR1 and ADAR2
33 expression change at RNA level was opposite to each other. That is, an increase of ADAR1
34 mRNA was noticed in human CHD cases, whereas ADAR2 mRNA was vastly down-regulated.
35 The increase in ADAR1 may be explained by the stress induced by CHD. The dramatic decrease
36 in ADAR2 in CHD cases was unexpected and prompted further investigation into its effects on
37 the heart. Therefore we performed expression analysis on a microarray data encompassing
38 ischemic and non-Ischemic cardiomyopathy patient myocardial tissues. A strong down-
39 regulation of ADAR2 was observed in both ischemic and especially non-ischemic cases.
40 However, ADAR1 showed a mild increase in the case of non-ischemic myocardial tissues. To
41 further explore the role of ADAR2 with respect to heart physiology. We selected a protein
42 coding gene filamin B (FLNB). FLNB is known to play an important role in heart development.
43 Although there were no observable changes in its expression, the editing levels of FLNB
44 dropped dramatically in ADAR2$^{-/-}$ mice. We also performed miRNA profiling from ADAR2 $^{-/-}$
45 mice heart tissue revealed a decrease in expression of miRNAs. It is established that aberrant
46 expression of these miRNAs is often associated with cardiac defects. This study proposes that
47 sufficient amounts of ADAR2 might play a vital role in preventing cardiovascular defects.

48

49 **KEYWORDS**

50 Congenital heart defect, ADAR1, ADAR2, Cardiomyopathy

51

52

53

54

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

55 **Introduction**

56 Congenital Heart Disease (CHD) is defined as structural or functional heart defect. It belongs to
57 a heterogeneous group of diseases and can be classified anatomically, clinically,
58 epidemiologically and developmentally (1-3). The most common types of CHDs among
59 hospitalized patients are VSD (Ventricular Septal Defects), TOF (Teratology of Fallot), PDA
60 (Patent Ductus Arteriosus), TGA (Transposition of Arteries), ASD (Atrial Septal Defect) and
61 CAVSD (Atrioventricular Septal Defect) (4). Recent studies indicate that 11% of Pakistani
62 children die due to cardiac anomalies at the first postnatal month (5).. Genetic conditions or
63 environmental factors such as maternal diabetes or rubella are identified in some cases but for
64 most babies born with a heart defect the cause remain unknown(6).

65 The multi-lineage differentiation during cardiogenesis is orchestrated by a precise spatial and
66 temporal regulation of gene expression. Genetic studies in humans and knockout embryos have
67 identified various genes, such as *TBX5, NKX2-5, GATA4, CX43, NOTCH1* and *VEGF*
68 responsible for sporadic and inherited CHD cases (7).

69 In humans, the most prevalent type of RNA editing is adenosine (A) to inosine (I) (8). This
70 complex post-transcriptional hydrolytic deamination reaction is carried out by adenosine
71 deaminase (ADAR) family of enzymes. This family acts on double stranded RNA and comprise
72 of three members ADAR1, ADAR2 and ADAR3. ADAR1 ad ADAR2 are actively involved in
73 adenosine deamination however, ADAR3 is non-functional (9). Different studies have shown
74 that the extent of RNA editing not only varies among individuals but also show high tissue
75 specificity. Approximately 2.5 million sites in human transcriptome undergo editing however a
76 vast majority of them lie in the Alu elements located mostly in the introns and UTR (untranslated
77 region) (10). However, the functional consequence of majority of RNA editing events still
78 remain elusive. RNA editing is known to modulate splicing, coding potential, transcript stability
79 and even alters the processing and targeting of the microRNAs (miRNA) (8, 11, 12). RNA
80 editing process affects RNA stability by conversion of a stable A:U base pair to a relatively
81 unstable I:U base pair followed by unwinding of the RNA duplex and making it susceptible to
82 single strand specific RNases (11). Recent study focusing on RNA editing events in six different
83 tissues have demonstrated an average of 79,976 editing sites in heart (10). Previous report
84 focusing on cyanotic congenital heart disease has indicated a significant decline in ADAR2 RNA

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

85 level but no prominent difference in ADAR1 expression. Moreover, they showed high editing of
86 MED13 in cyanotic CHD cases as opposed to acyanotic CHD patients (13). Recent study has
87 shown an increase in expression of a lysosomal cysteine protease encoded by cathepsin S RNA
88 (CTSS) via ADAR1 mediated RNA editing followed by HuR recruitment . Cathepsin S has a
89 role in vascular inflammatory processes and the CTSS mRNA editing is increased in hypoxic or
90 pro-inflammatory conditions as wells as in patients suffering from clinical or subclinical vascular
91 damage (14).

92 In the current study we have determined the RNA level of ADAR1 and ADAR2 in congenital
93 heart disease patients. We also checked the relative gene expression of FoxP1 which is an
94 important transcription factor crucial for angiogenesis. We found a strong down-regulation of
95 ADAR2 and an up-regulation of ADAR1. Interestingly, microarray data analysis of human non-
96 ischemic myocardial tissues showed similar trend. Interestingly the ischemic myocardial tissues
97 showed completely opposite trend. To further explore the role of ADAR2 in heart physiology,
98 we used ADAR2 knockout mouse. Although no strong anomaly in heart physiology was
99 observed as documented previously (15) however, we found down-regulation of different
100 microRNAs.

101

102 ## Materials and Methods

103 ### Collection of samples

104 The blood samples were collected from 35 patients displaying different congenital heart defects
105 from RIC (Rawalpindi institute of cardiology), stored in ice during transportation. Samples
106 were segregated on the basis of age (3 months-16 years) and sex. Patient's echocardiography
107 reports were consulted to confirm the presence of congenital heart defects and all sample
108 collection was done pre-operatively. Whereas 13 control samples were collected from healthy
109 individuals using same parameters. Interviews were conducted personally using the specified
110 questionnaires. Information on age, gender, medication and family history was recorded.
111 Perspective study was initiated after getting approval from ethical committees of both CIIT and
112 collaborating hospitals.

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

113 **cDNA synthesis**

114 Five cubic centimeters of whole blood was collected from each patient in ethylenediamine
115 tetraacetic acid (EDTA) test-tubes. To avoid RNA degradation, blood was kept at 4ºC up to 24 h
116 following collection before RNA was extracted. RNA extraction was carried out from peripheral
117 blood mono-nuclear cells using the TRIzol ® LS Reagent (Invitrogen, Germany) according to
118 manufacturer's instruction. Optical density of the RNA was measured immediately following
119 extraction. RNA samples showing A260/280 below 1.8 or above 2.0 were not taken for further
120 analysis. One microgram of RNA was used for production of complementary DNA (cDNA)
121 using Revert aid first strand cDNA synthesis kit (Thermo scientific, USA). A negative control
122 was set up against each of the sample that lacked Reverse transcriptase and was termed as –RT
123 (minus Reverse Transcriptase).

124 **Real time PCR**

125 The relative mRNA expression of genes were examined using a quantitative PCR with gene
126 specific primer sets (IDT,USA and Macrogen, South Korea) and *TUBB11* was taken as internal
127 control. 5x HOT FIREPol® EvaGreen®qPCR Mix plus (ROX) (Solis Bio Dyne, Tartu, Estonia)
128 master mixed was used for qPCR reaction. Sequence was taken from ensemble and primers were
129 synthesized by Integrated DNA Technology (**biotools.idtdna.com/Primer Quest).** The primers
130 are listed in the supplementary table 1

131 **Statistical analysis**

132 Statistical analyses were performed with Graph-pad Prism 7.0b. For expression data, the target
133 genes (*ADAR1, ADAR2, FOXP1*) CT was normalized with the control gene (*TUB1*) Ct.
134 Depending on experiment, the statistical significance was determined using the Mann-Whitney
135 test) with $P < .05$ considered significant.

136

137 **MicroArray analysis**

138 Micro array data analysis was performed using CARMA web tool (16) on the GEO data set
139 GDS1362 (17)focusing on expression analysis from myocardial tissues of non-ischemic (NICM),

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

140 Ischemic cardiomyopathy (ICM) patients as opposed to non-failing heart tissues. The raw micro
141 array data was extracted and normalization of the data was performed by gcRMA package.

142 **Mice**

143 The *Adar2*$^{-/-}$ knockout mouse was a kind gift of Peter Seeburg. These transgenic mice are in an
144 SV129 background. As ADAR2 deficiency leads to early postnatal lethality, the mice were
145 rescued with a pre-edited Gria2 receptor (*Gria2*$^{R/R}$) (18, 19). Mice were bred in Vienna Biocentre
146 facility animal house. *Gria2*$^{R/R}$*; ADAR2*$^{+/-}$ were intercrossed. The resulting sibling female
147 offspring of genotype *Gria2*$^{R/R}$*; Adar2*$^{-/-}$ and *Gria2*$^{R/R}$*; ADAR2*$^{+/+}$ was euthanized at the age of
148 post natal day 6 (P6). Whole heart was dissected and subsequently used for RNA preparation
149 from three biological replicates (18, 20).

150

151 **RNA extraction and miRNA cloning**

152 Female mouse whole heart as dissected at the age of post natal day 6 (P6), homogenized and
153 total RNA was extracted using TriFast reagent according to manufacturer's instructions
154 (PEQLAB Biotechnologie GmbH, Erlangen, Germany). miRNA library preparation was
155 performed as previously described (21).

156

157 **Sequencing and clipping of reads**

158 Completed libraries were quantified with the Agilent Bioanalyzer dsDNA 1000 assay kit and
159 Agilent QPCR NGS library quantification kit. Cluster generation and sequencing was carried out
160 using the Illumina Genome Analyzer IIx system according to the manufacturer's guidelines.
161 Illumina sequencing was performed at the CSF NGS Unit (csf.ac.at). After sequencing at a read
162 length of 36 base pairs, adaptor sequences were removed using Cutadapt (22).

163 **Mapping to mature miRNA sequences**

164 Mapping of clipped reads to mature miRNA sequences was performed as described previously
165 Mapping was performed using NextGenMap, restricting the mapped reads to have at least 90%
166 identity (# differences/alignment length) (23).

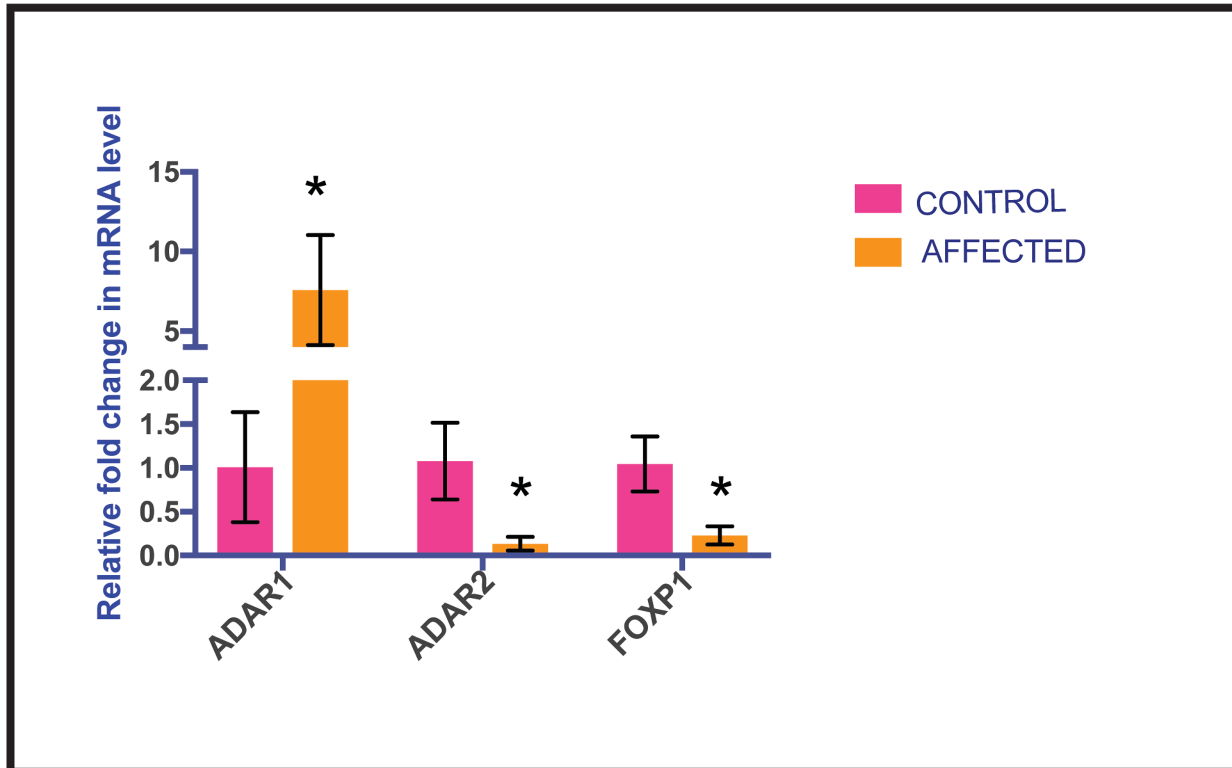
bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

167 # Results

168 **ADAR2 has the lowest expression in CHD patients**

169 RNA was extracted from 35 affected congenital heart disease patients and 13 normal individuals
170 were taken as control. Most of the patients had VSD (Ventricular septal defects). All CHD
171 samples were pre-operative cases. Since recent reports indicated, that RNA editing might be
172 involved in cardiogenisis (13, 14). We checked the expression of the functional RNA editing
173 enzymes using quantitative realtime PCR (qPCR). A significant decline in ADAR2 expression
174 was observed. However, on the contrary, ADAR1 showed a significant increase (Figure 1a). The
175 observed elevated ADAR1 expression is in line with the recent finding demonstrating higher
176 ADAR1 expression in the human patients undergoing carotid endarterectomy operation(14). As a
177 control for CHD, we selected a member of fork head box family of transcription factor (FoxP1).
178 FoxP1 has a critical role in murine as well as human heart development. It showed high
179 expression during embryonic stages as opposed to postnatal stage and is critical for
180 cardiomyocyte proliferation (24). We found a significant decrease in FoxP1 expression
181 confirming that the RNA extracted from the blood of CHD patients can depict the expression
182 differences between normal and patient samples. Surprisingly out of these three genes, ADAR2
183 was strongly down-regulated (Figure 1) pointing towards its expression modulation in relation to
184 heart disease.

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



185

186 **Figure 1. ADAR2 has the lowest expression in CHD patients:** Bar graph showing relative
187 RNA level of ADAR1, ADAR2 and FOXP1 as opposed to control. ADAR2 and FOXP1 show a
188 significant reduced expression at RNA level whereas ADAR1 shows upregulation. $*P < 0.05$
189 (versus control) was determined by Mann–Whitney *U* test.

190

191 **Heart defect specific function**

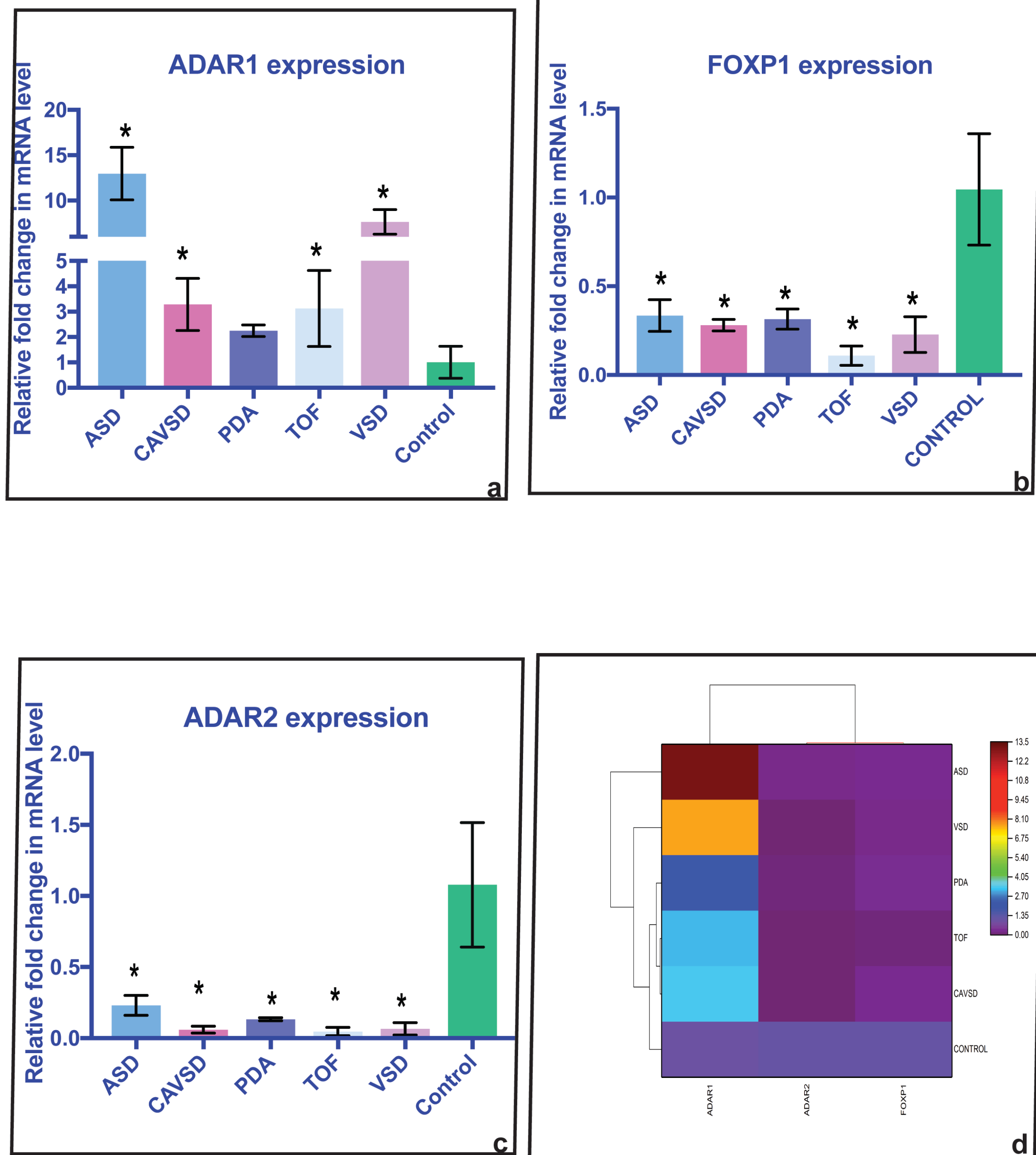
192 We further investigated whether this increase in ADAR1 is specific for a heart defect. As in our
193 study the patients were suffering from different forms of congenital heart disease. ADAR1 was
194 strongly up-regulated in ASD followed by VSD. However, it shows approximately 3fold
195 increase- in TOF and CAVSD (Figure 2a and d). On the contrary, ADAR2 shows a strong
196 significant decline in CAVSD, TOF and VSD. (Figure 2b and d). This specificity of gene
197 expression of ADAR1 and ADAR2 with heart defects can be answered by the differences in the
198 cardiac myocytes. Atrial, ventricular and nodal cells are morphologically, molecularly and
199 functionally distinct. We propose that ADAR1 might have a more critical function in atrium
200 development as compared to ventricles whereas ADAR2 might be more crucial for ventricular

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

201 development. In the case of FOXP1, the most prominent decline was observed in the TOF
202 patient samples (Figure 2c and d). FOXP1 plays a critical role in maintaining a balance of
203 cardiomyocyte proliferation and differentiation via regulation of Fgf ligand and modulation of
204 Nkx2.5 expression (25).This might answer the observed an increase in ADAR1 mRNA level and
205 a strong decrease in FOXP1 expression.

206

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



207

208 **Figure 2**. **Heart defect specific function a.** Bar graph showing up-regulation of ADAR1 in
209 different CHD cases. ADAR1 is significantly up-regulated in ASD and VSD. However ,3-4 fold
210 increase at RNA level was found in AVSD and TOF cases. The PDA cases did not show a
211 significant change. *$P < 0.05$ (versus control) was determined by Mann–Whitney *U* test.

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

212 **b.** Bar graph showing strong decline in ADAR2 expression particularly in AVSD, TOF and
213 VSD. ADAR2 is significantly down-regulated in all CHD cases. *$P < 0.05$ (versus control) was
214 determined by Mann–Whitney *U* test.

215 **c.** Bar graph showing reduced expression of FOXP1 in CHD cases. The strongest decline was
216 observed in TOF samples. *$P < 0.05$ (versus control) was determined by Mann–Whitney *U* test.

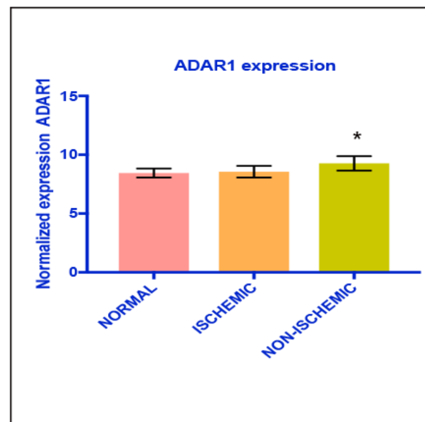
217 **d.** Heat map showing expression level of ADAR1, ADAR2 and FOXP1 as opposed to control in
218 different CHD cases.

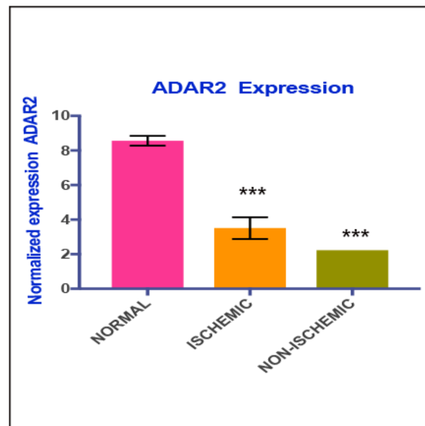
219

220 **ADAR1, ADAR2 and Cardiomyopathy**

221 Since all the above-mentioned results, were observed only in PBMCs we performed microarray
222 data analysis on the Geo Dataset GDS1362 (17). This dataset comprised of differentially
223 expressed genes in myocardial tissues from non-failing heart, ischemic and non-ischemic
224 cardiomyopathy patients. We found a similar trend of expression of ADAR1 and ADAR2 in
225 myocardial tissues from non-ischemic patients. ADAR1 showed a slight but significant increase
226 whereas ADAR2 showed a strong decline in expression. Interestingly we found that the
227 decrease in expression of ADAR2 was more prominent in non-ischemic as opposed to ischemic
228 myocardial tissues (Figure 3).

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.





229

230 **Figure 3 (a)** Bar graph showing slight increase in ADAR1 expression in ischemic myocardial
231 tissues from cardiomyopathy patients.

232 **(a)** Bar graph showing significant decrease in ADAR2 expression in ischemic and non-ischemic
233 myocardial tissues from cardiomyopathy patients.

234 *$P < 0.05$ (versus control) was determined by Mann–Whitney U test.

235

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

Filamins and Cardiac Defects

The strong decrease of ADAR2 expression in CHD patients made us curious to further investigate what happens in ADAR2$^{-/-}$ mice heart?. Actin binding proteins such as FLNA and FLNB play essential role in the vascular development. FLNA is ubiquitously expressed whereas FLNB expression is mainly in the endothelial cells (26). Complete loss of FLNA results in severe structural defects in the heart involving atria, ventricles and outflow tracts (27). The decrease in ADAR2 mRNA level in CHD patients has urged us to look for protein coding targets that play significant role in cardiac development. Therefore, we chose FLNB. FLNA has been previously reported as ADAR2 editing target in the heart. Editing of FLNA drops down dramatically in ADAR2$^{-/-}$mice heart (28). FLNB shows highest editing in the heart as compared to other tissues (28). Therefore, we checked the expression and editing of FLNB in the absence of ADAR2. We observed a dramatic decrease of 24% in editing of FLNB in ADAR2$^{-/-}$ mice heart (Figure 4).

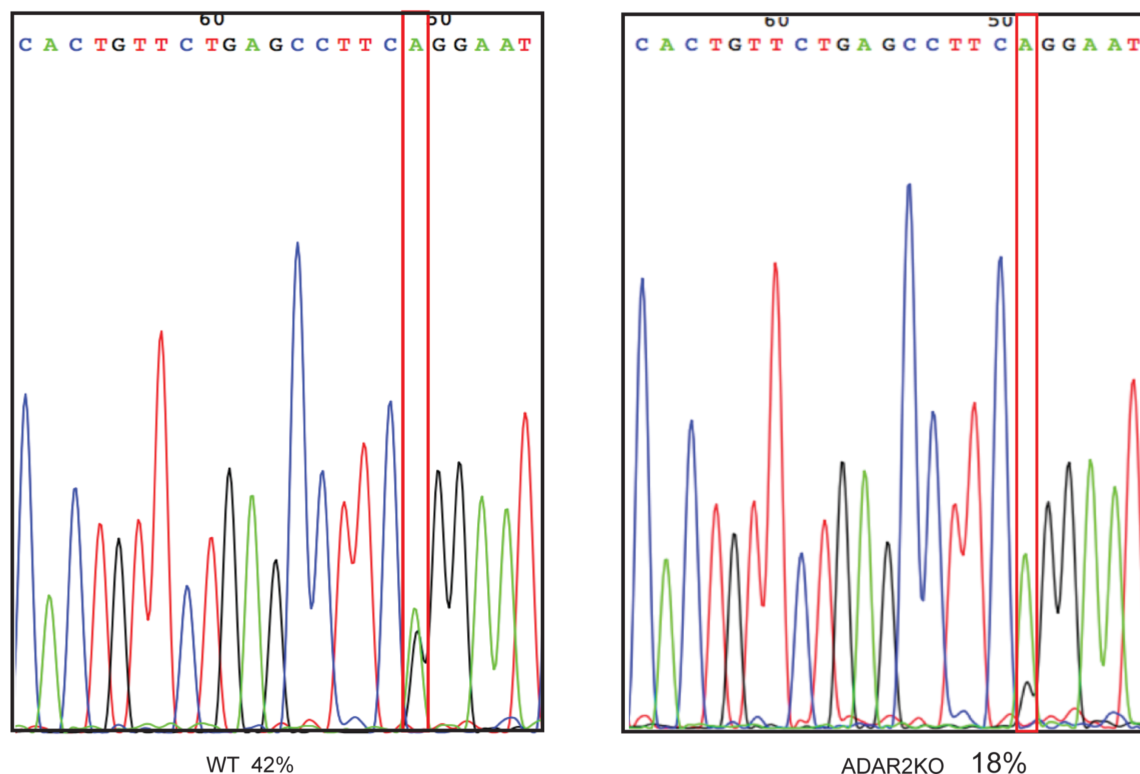
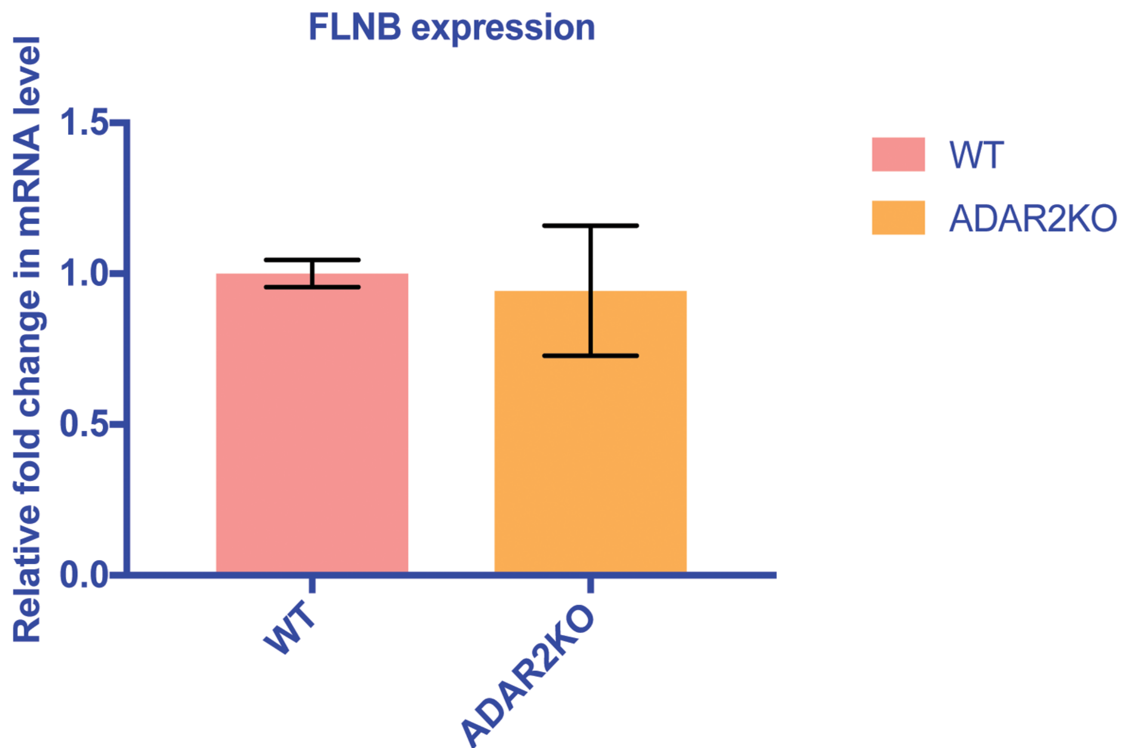


Figure 4. **FLNB editing in ADAR2 KO mouse:** Electropherogram showing 24% decrease in editing as compared to WT mouse

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

252 To our surprise, we did not observe any change in expression of FLNB in the ADAR2$^{-/-}$ mice
253 heart as compared to wild type mice heart (Figure 5). This indicates that edited FLNB might
254 have a specific function.



255
256 **Figure 5. FLNB expression in the ADAR2 KO mouse heart:** Bar graph showing no
257 significant change in expression of FLNB in the heart on ADAR2

258

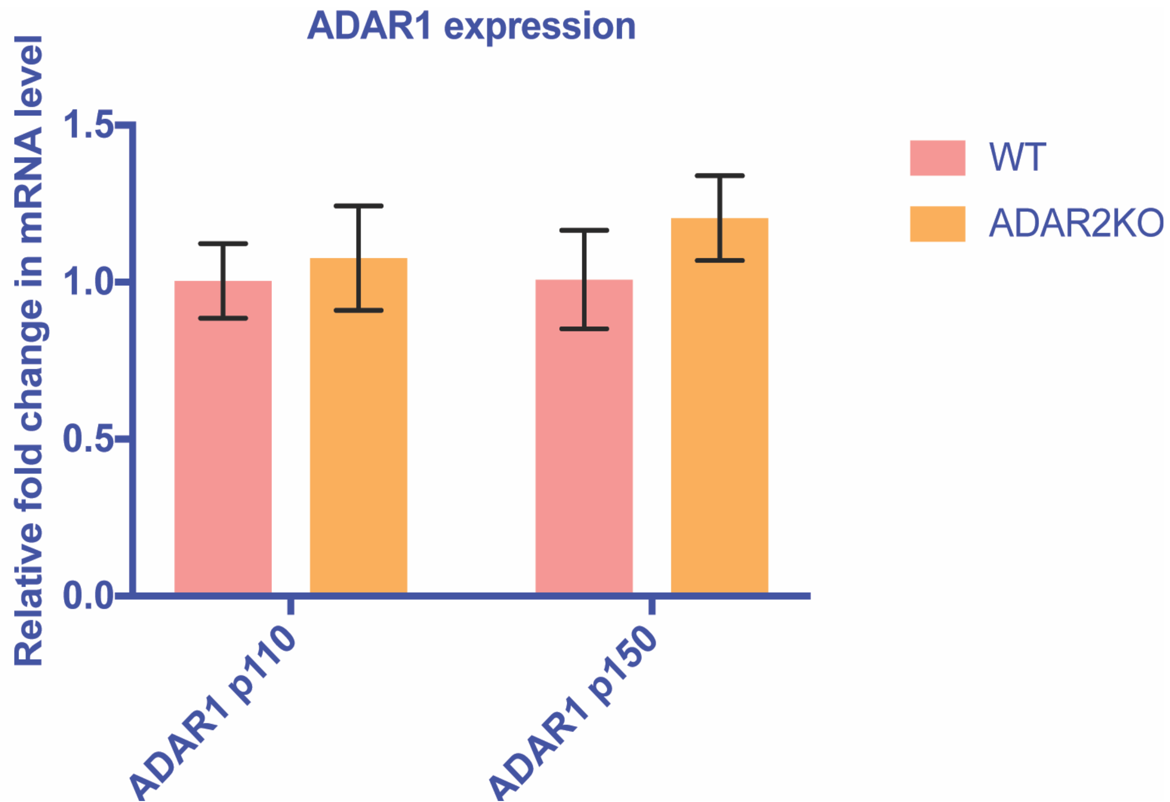
259 **ADAR1 expression in ADAR2 KO mouse heart**

260 Since increase in ADAR1 has been found in CHD patients. We determined whether this
261 observed increase in ADAR1 is due to deregulation of ADAR2. Therefore, we determined
262 ADAR1 level in the absence of ADAR2. We did not find any significant change in ADAR1 level
263 (Figure 6). Therefore, we can conclude that the observed increase in ADAR1 is soley because of
264 the CHD defect.

265

266

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



267

268

269 **Figure 6**. **ADAR1 isoforms expression in ADAR2 KO mouse:** Bar graph showing no
270 significant change in expression of ADAR1 isoforms (p110 and p150) in the heart on ADAR2

271

272 **ADAR2$^{-/-}$ heart shows down-regulation of miR-29b**

273 A previous study focusing on ADAR2 $^{-/-}$ mice showed a statistically significant decrease in heart
274 rate (15). We performed RNA sequencing of ADAR2$^{-/-}$ mice heart samples in triplicates and
275 observed approximately ~2-fold decrease in miR-29b level consistently at P6 stage.
276 Approximately 1.5 fold down-regulation has been observed for miR451-b, miR451-a, miR19b,
277 (Table 1). To our surprise, we did not observe any up-regulated miRNAs. Quantitative trait loci
278 (QTLs) associated with miR-29 a and b show their potential involvement in cardiac diseases
279 (29). miR-29 family shows strong expression in lung, kidney and the heart. It expresses
280 predominantly approximately 5-12 folds in cardiac fibroblasts as compared to cardiomyocytes.
281 Moreover, the miR-29 family is down-regulated in fibrotic scars after myocardial infarction and

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

282 can lead to cardiac fibrosis by boosting collagen expression. miR29-b has an antifibrosis role as
283 it targets promoters of several extracellular matrix genes (30). Recent reports have documented a
284 cardioprotective role of miR29-b. miR 29b inhibits angiotensin II induced cardiac fibrosis by
285 targeting TGF- ß /Smad3 pathway (31).

286 After miR-29b, miR-451(a and b), miR-19b1and different members of let-7, family also showed
287 down-regulation in ADAR2 knockout mice heart However, they showed significant but small
288 down-regulation of only about 1-1.5 fold (Table -1). Aberrant expression of let-7 family has been
289 linked to diverse cardiovascular diseases such as fibrosis, hypertrophy, dilated cardiomyopathy
290 (DM), myocardial infarction (MI), atherosclerosis and hypertension (32). This down regulation
291 of microRNAs on ADAR2 knock-out point towards a potential regulatory mechanism mediated
292 by ADAR2 in the heart development and physiology.

293

Sr.No.	**miRNA**	**Log2 Fold change**	**Pvalue**	**Reference**
1	miR-29b	-1.97	0.001599	(33)
2	miR-451b	-1.51	0.005	(34)
3	miR-451a	-1.51	0.005	(34)
4	miR-19b1	-1.48	0.015	(33)
5	let7c-2	-1.47	0.0017	(35)
6	let7c-1	-1.39	0.003	(35)
7	let7-i	-1.23	0.01	(35)
8	let7-b	-1.23	0.01	(35)
9	miR-382	-1.21	0.04	
10	miR-26a	-1.19	0.03	
11	miR-378	-1.19	0.011	(36)
12	miR-378a	-1.14	0.019	(36)
13	miR-130a	-1.09	0.024	(37)

294

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

295 **Table 1.** miRNAs significantly downregulated in ADAR2$^{-/-}$ mice heart as compared to wild type

296

297 Discussion

298 Adenosine deamination by ADARs is a post-transcriptional event that can diversify the
299 transcripts both at sequence as well as structure level. The deregulation of editing has been
300 associated with number of diseases (8, 13, 33).

301 ADARs play a significant role in development. Moreover the tissue and site specific editing
302 largely affects the differential expression of substrate transcripts (38, 39). In our study, we found
303 a strong down-regulation of ADAR2 and a strong increase in ADAR1 in ASD and VSD patient
304 samples. This observation is in line with previous finding demonstrating increased CTSS mRNA
305 editing due to up-regulation of ADAR1 in human atherosclerotic plaques (14). Since the
306 expression analysis was performed only on the PBMCs from normal and CHD patients we
307 further extended our study to myocardial tissues. Expression analysis of myocardial tissues from
308 ischemic and non-ischemic patients showed a significant decline in ADAR2 expression level
309 (Figure 3). This result supported our finding that ADAR2 not only down-regulates in PBMCS
310 but also showed decreased expression in human cardiomyopathy tissues.

311 The stong downregulation of ADAR2 with respect to heart disease urged us to further explore
312 what heart related processes might be ADAR2 regulating? To address this query, we used
313 ADAR2 knock out mouse. We chose FLNB which plays an essential role in the heart however
314 lack of ADAR2 strongly decreased its editing. We did not observe any change in FLNB
315 expression in the absence of ADAR2. The increase on ADAR1 in the PBMCs and the
316 simultaneous downregulation of ADAR2 posed a question whether the observed effect is
317 because of ADAR2 or is it the consequence of CHD? We observed no significant change in
318 expression of the ADAR1 isoforms in the absence of ADAR2. This shows that CHD might be
319 triggering an inflammatory response leading to increase in ADAR1. The elevated ADAR1
320 expression in atherosclerosis has been documented previously (14).

321 ADARs can modulate microRNA processing and also are capable of retargeting the microRNA
322 to different substrate (35). Like ADAR1, ADAR2 also can modulate microRNA processing.
323 Since a number of micro RNAs like miR-1, miR-423 are associated with heart disease we

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

324 thought of investigating the microRNA profile in ADAR2 knock out mouse heart (40).
325 Surprisingly, we did not observe any up-regulated micro RNAs in the ADAR2$^{-/-}$ mice heart.
326 However, we observed a decline of ~1.5-2 fold in miR-29b, miR-451, miR19 and members of let
327 -7 family (Table 1).

328 miR-29b family regulates a plethora of proteins at RNA level that are involved in cardiac
329 fibrosis. This family has highest expression in the heart fibroblast population and comprises of
330 three members miR-29a, miR-29b and miR-29c. miR-29b differs only by one base from miR-
331 29a and miR-29c. Among the three members, miR-29b expresses strongly in cardiac fibroblasts
332 as opposed to miR-29a and miR-29c.(30). Angiotensin-II (Ang-II) induced hypertensive cardiac
333 fibrosis in-vivo and in-vitro is associated with cardiac fibrosis. miR-29b inhibits activation
334 ERK1/2 by preventing its phosphorylation. miR-29b suppresses the TGF-β /Smad-ERK-MAP
335 kinase crosstalk (31). Two members of miR-451 a and b show down-regulation by 1.5 fold. miR-
336 451is down-regulated in the heart tissues from hypertrophic cardiomyopathy patients. It directly
337 targets tuberous sclerosis complex 1 (TSC1) and inhibits formation of auto-phagosomes and
338 cardiac hypertrophy (41). Another microRNA, miR-19b belongs to miR-17-92 cluster. The
339 decreased expression of miR-19b is correlated with cardiovascular diseases (42). miR-19b
340 overexpression promotes differentiation by stimulating cell proliferation and inhibiting Wnt-β-
341 catenin pathway consequently blocking apoptosis of cardiac P19 cells(43).

342 Apart from the above mentioned microRNAs, some members of let7 family like let7-c, let-7-i
343 and let7-b are down-regulated approximately 1.2-1.4 fold. The human let-7 family comprises of
344 13 members. Let7-c is elevated in endothelial to mesenchymal transition (EndMT) (32). It
345 shows reduced expression in coronary artery disease patients as opposed to control (44). Let7-i
346 limits the toll like receptors like TLR4 by targeting it. (45) and is down-regulated in dilated
347 cardiomyopathy (32).

348 Most of the down-regulated microRNAs on ADAR2 knock down in our study are related with
349 cardiovascular disorders. This implies that ADAR2 might have a cardio-protective function as
350 these microRNAs are mostly reduced in different cardiac defects. The increase in ADAR1 in
351 CHD cases is in line with previous finding of elevated ADAR1 expression in endothelial cells
352 stress response and atherosclerosis (14).

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

353 **Acknowledgments**

354 We thank Peter Seeburg (MPI, Heidelberg) for the kind gift of Adar2$^{-/-}$ knockout mice and
355 Dr.Michael Jantsch (Medical University, Vienna) for supporting the microRNA work. This work
356 was supported by the Higher Education Commission startup grant IPFP/HRD/HEC/2014/1622.
357 We thank Kate Middleton, for reviewing the manuscript.

358

359

360

361 **References**

362 1. Hobbs CA, Cleves MA, Melnyk S, Zhao W, James SJ. Congenital heart defects and abnormal
363 maternal biomarkers of methionine and homocysteine metabolism. The American journal of clinical
364 nutrition. 2005;81(1):147-53.
365 2. Botto LD, Mulinare J, Erickson JD. Do multivitamin or folic acid supplements reduce the risk for
366 congenital heart defects? Evidence and gaps. American Journal of Medical Genetics Part A.
367 2003;121(2):95-101.
368 3. Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J, et al.
369 Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. BJOG: An International
370 Journal of Obstetrics & Gynaecology. 2006;113(12):1412-8.
371 4. Al-Hamash SM. Pattern of congenital heart disease: a hospitalbased study. Al Kindy College
372 Medical Journal. 2006;3:44-8.
373 5. Pathan IH, Bangash SK, Khawaja AM. SPECTRUM OF HEART DEFECTS IN CHILDREN PRESENTING
374 FOR PAEDIARIC CARDIAC SURGERY. Pakistan Heart Journal. 2016;49(1).
375 6. Botto LD, Lynberg MC, Erickson JD. Congenital heart defects, maternal febrile illness, and
376 multivitamin use: a population-based study. Epidemiology. 2001;12(5):485-90.
377 7. Bruneau BG. The developmental genetics of congenital heart disease. Nature.
378 2008;451(7181):943-8.
379 8. Tariq A, Jantsch MF. Transcript diversification in the nervous system: A to I RNA-editing in CNS
380 function and disease development. Frontiers in neuroscience. 2012;6:99.
381 9. Tariq A, Jantsch MF. Transcript diversification in the nervous system: A to I RNA editing in CNS
382 function and disease development. Frontiers in neuroscience. 2012;6.
383 10. Picardi E, Manzari C, Mastropasqua F, Aiello I, D'Erchia AM, Pesole G. Profiling RNA editing in
384 human tissues: towards the inosinome Atlas. Scientific reports. 2015;5:14941.
385 11. Wang IX, So E, Devlin JL, Zhao Y, Wu M, Cheung VG. ADAR regulates RNA editing, transcript
386 stability, and gene expression. Cell reports. 2013;5(3):849-60.
387 12. Nishikura K. A-to-I editing of coding and non-coding RNAs by ADARs. Nature reviews Molecular
388 cell biology. 2016;17(2):83-96.
389 13. Borik S, Simon AJ, Nevo-Caspi Y, Mishali D, Amariglio N, Rechavi G, et al. Increased RNA editing
390 in children with cyanotic congenital heart disease. Intensive care medicine. 2011;37(10):1664.
391 14. Stellos K, Gatsiou A, Stamatelopoulos K, Matic LP, John D, Lunella FF, et al. Adenosine-to-inosine
392 RNA editing controls cathepsin S expression in atherosclerosis by enabling HuR-mediated post-
393 transcriptional regulation. Nature Medicine. 2016.

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

394 15. Horsch M, Seeburg PH, Adler T, Aguilar-Pimentel JA, Becker L, Calzada-Wack J, et al.
395 Requirement of the RNA-editing enzyme ADAR2 for normal physiology in mice. Journal of Biological
396 Chemistry. 2011;286(21):18614-22.
397 16. Rainer J, Sanchez-Cabo F, Stocker G, Sturn A, Trajanoski Z. CARMAweb: comprehensive R-and
398 bioconductor-based web service for microarray data analysis. Nucleic acids research.
399 2006;34(suppl_2):W498-W503.
400 17. Kittleson MM, Minhas KM, Irizarry RA, Ye SQ, Edness G, Breton E, et al. Gene expression analysis
401 of ischemic and nonischemic cardiomyopathy: shared and distinct genes in the development of heart
402 failure. Physiological genomics. 2005;21(3):299-307.
403 18. Brusa R, Zimmermann F, Koh D-S, Feldmeyer D, Gass P, Seeburg PH, et al., editors. Early-onset
404 epilepsy and postnatal lethality associated with an editing-deficient GluR-B allele in mice. Cold Spring
405 Harbor Symp Quant Biol; 1995: JSTOR.
406 19. Higuchi M, Maas S, Single FN, Hartner J, Rozov A, Burnashev N, et al. Point mutation in an AMPA
407 receptor gene rescues lethality in mice deficient in the RNA-editing enzyme ADAR2. Nature.
408 2000;406(6791):78-81.
409 20. Hartner JC, Schmittwolf C, Kispert A, Müller AM, Higuchi M, Seeburg PH. Liver disintegration in
410 the mouse embryo caused by deficiency in the RNA-editing enzyme ADAR1. Journal of Biological
411 Chemistry. 2004;279(6):4894-902.
412 21. Vesely C, Tauber S, Sedlazeck FJ, von Haeseler A, Jantsch MF. Adenosine deaminases that act on
413 RNA induce reproducible changes in abundance and sequence of embryonic miRNAs. Genome research.
414 2012;22(8):1468-76.
415 22. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads.
416 EMBnet journal. 2011;17(1):pp. 10-2.
417 23. Sedlazeck FJ, Rescheneder P, Von Haeseler A. NextGenMap: fast and accurate read mapping in
418 highly polymorphic genomes. Bioinformatics. 2013;29(21):2790-1.
419 24. Chang SW, Mislankar M, Misra C, Huang N, DaJusta DG, Harrison SM, et al. Genetic
420 abnormalities in FOXP1 are associated with congenital heart defects. Human mutation.
421 2013;34(9):1226-30.
422 25. Zhang Y, Li S, Yuan L, Tian Y, Weidenfeld J, Yang J, et al. Foxp1 coordinates cardiomyocyte
423 proliferation through both cell-autonomous and nonautonomous mechanisms. Genes & development.
424 2010;24(16):1746-57.
425 26. del Valle-Pérez B, Martínez VG, Lacasa-Salavert C, Figueras A, Shapiro SS, Takafuta T, et al.
426 Filamin B plays a key role in vascular endothelial growth factor-induced endothelial cell motility through
427 its interaction with Rac-1 and Vav-2. Journal of Biological Chemistry. 2010;285(14):10748-60.
428 27. Feng Y, Chen MH, Moskowitz IP, Mendonza AM, Vidali L, Nakamura F, et al. Filamin A (FLNA) is
429 required for cell–cell contact in vascular development and cardiac morphogenesis. Proceedings of the
430 National Academy of Sciences. 2006;103(52):19836-41.
431 28. Stulić M, Jantsch MF. Spatio-temporal profiling of Filamin A RNA-editing reveals ADAR
432 preferences and high editing levels outside neuronal tissues. RNA biology. 2013;10(10):1611-7.
433 29. Arnold N, Koppula PR, Gul R, Luck C, Pulakat L. Regulation of cardiac expression of the diabetic
434 marker microRNA miR-29. PLoS One. 2014;9(7):e103284.
435 30. Van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, et al.
436 Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis.
437 Proceedings of the National Academy of Sciences. 2008;105(35):13027-32.
438 31. Zhang Y, Huang X-R, Wei L-H, Chung AC, Yu C-M, Lan H-Y. miR-29b as a therapeutic agent for
439 angiotensin II-induced cardiac fibrosis by targeting TGF-β/Smad3 signaling. Molecular Therapy.
440 2014;22(5):974-85.

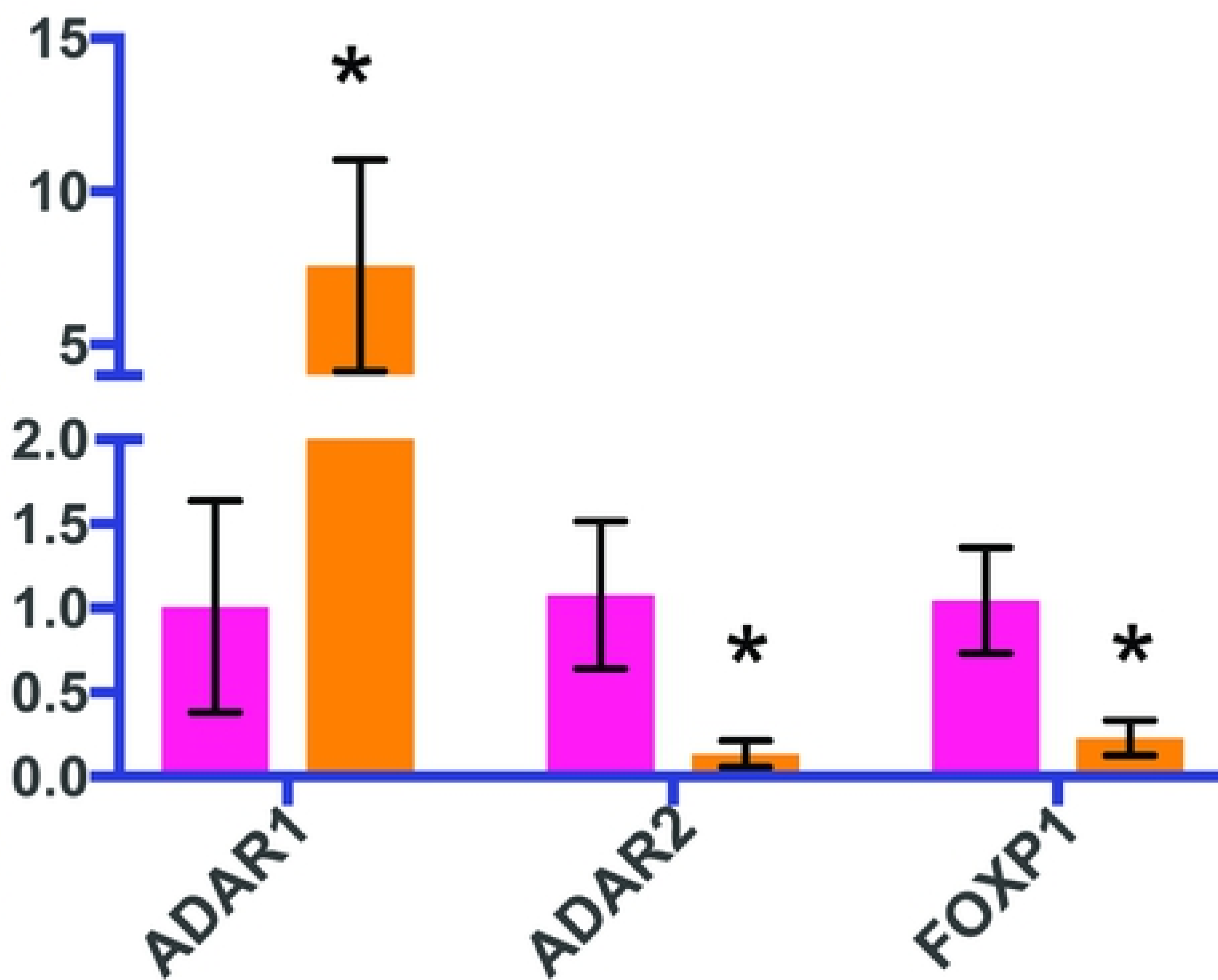
bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

441 32. Bao M-H, Feng X, Zhang Y-W, Lou X-Y, Cheng Y, Zhou H-H. Let-7 in cardiovascular diseases, heart
442 development and cardiovascular differentiation from stem cells. International journal of molecular
443 sciences. 2013;14(11):23086-102.
444 33. Tomaselli S, Galeano F, Alon S, Raho S, Galardi S, Polito VA, et al. Modulation of microRNA
445 editing, expression and processing by ADAR2 deaminase in glioblastoma. Genome biology. 2015;16(1):5.
446 34. Liu D, Liu C, Wang X, Ingvarsson S, Chen H. Universal RNA editing in a human liver at the fetal
447 stage. 2012.
448 35. Chawla G, Sokol NS. ADAR mediates differential expression of polycistronic microRNAs. Nucleic
449 acids research. 2014;42(8):5245-55.
450 36. Vesely C, Tauber S, Sedlazeck FJ, Tajaddod M, Haeseler Av, Jantsch MF. ADAR2 induces
451 reproducible changes in sequence and abundance of mature microRNAs in the mouse brain. Nucleic
452 acids research. 2014;42(19):12155-68.
453 37. Ekdahl Y, Farahani HS, Behm M, Lagergren J, Öhman M. A-to-I editing of microRNAs in the
454 mammalian brain increases during development. Genome research. 2012;22(8):1477-87.
455 38. Huntley MA, Lou M, Goldstein LD, Lawrence M, Dijkgraaf GJ, Kaminker JS, et al. Complex
456 regulation of ADAR-mediated RNA-editing across tissues. BMC genomics. 2016;17(1):61.
457 39. Tariq A, Garncarz W, Handl C, Balik A, Pusch O, Jantsch MF. RNA-interacting proteins act as site-
458 specific repressors of ADAR2-mediated RNA editing and fluctuate upon neuronal stimulation. Nucleic
459 acids research. 2012;41(4):2581-93.
460 40. Paul D, Sinha AN, Ray A, Lal M, Nayak S, Sharma A, et al. A-to-I editing in human miRNAs is
461 enriched in seed sequence, influenced by sequence contexts and significantly hypoedited in
462 glioblastoma multiforme. Scientific Reports. 2017;7.
463 41. Song L, Su M, Wang S, Zou Y, Wang X, Wang Y, et al. MiR-451 is decreased in hypertrophic
464 cardiomyopathy and regulates autophagy by targeting TSC1. Journal of cellular and molecular medicine.
465 2014;18(11):2266-74.
466 42. van Almen GC, Verhesen W, van Leeuwen RE, van de Vrie M, Eurlings C, Schellings MW, et al.
467 MicroRNA-18 and microRNA-19 regulate CTGF and TSP-1 expression in age-related heart failure. Aging
468 cell. 2011;10(5):769-79.
469 43. Qin D-N, Qian L, Hu D-L, Yu Z-B, Han S-P, Zhu C, et al. Effects of miR-19b overexpression on
470 proliferation, differentiation, apoptosis and Wnt/β-catenin signaling pathway in P19 cell model of
471 cardiac differentiation in vitro. Cell biochemistry and biophysics. 2013;66(3):709-22.
472 44. Faccini J, Ruidavets J-B, Cordelier P, Martins F, Maoret J-J, Bongard V, et al. Circulating miR-155,
473 miR-145 and let-7c as diagnostic biomarkers of the coronary artery disease. Scientific Reports. 2017;7.
474 45. Chen X-M, Splinter PL, O'Hara SP, LaRusso NF. A cellular micro-RNA, let-7i, regulates Toll-like
475 receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium
476 parvum infection. Journal of Biological Chemistry. 2007;282(39):28929-38.

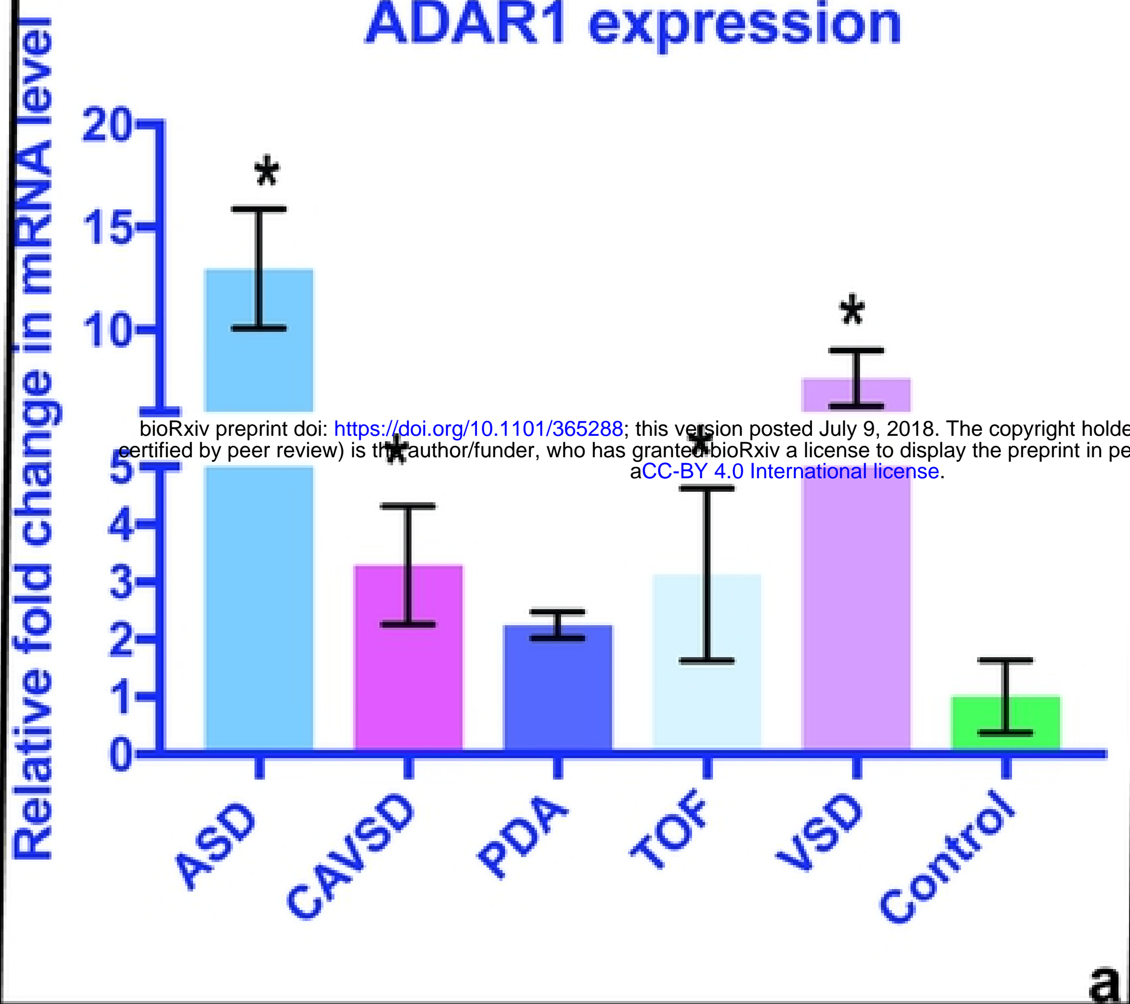
477

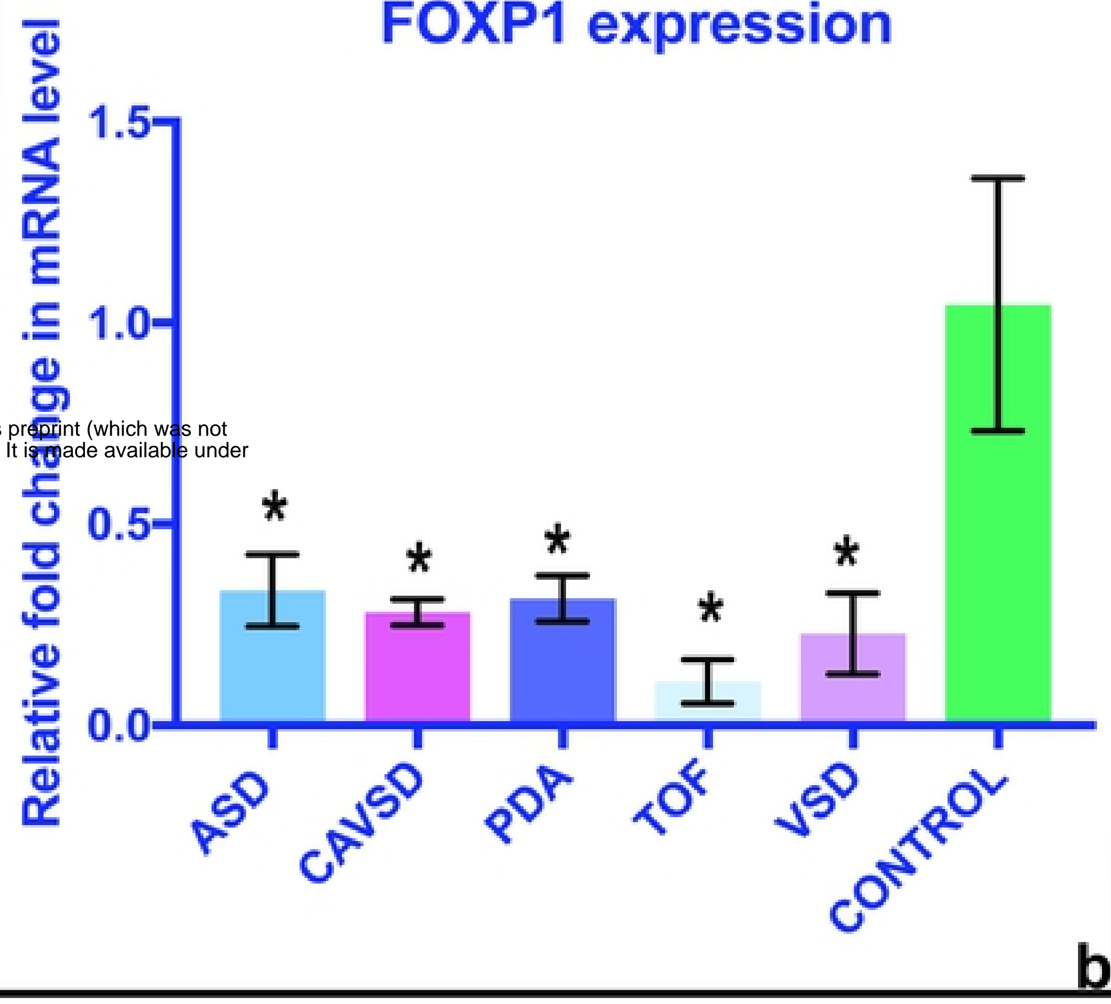
478

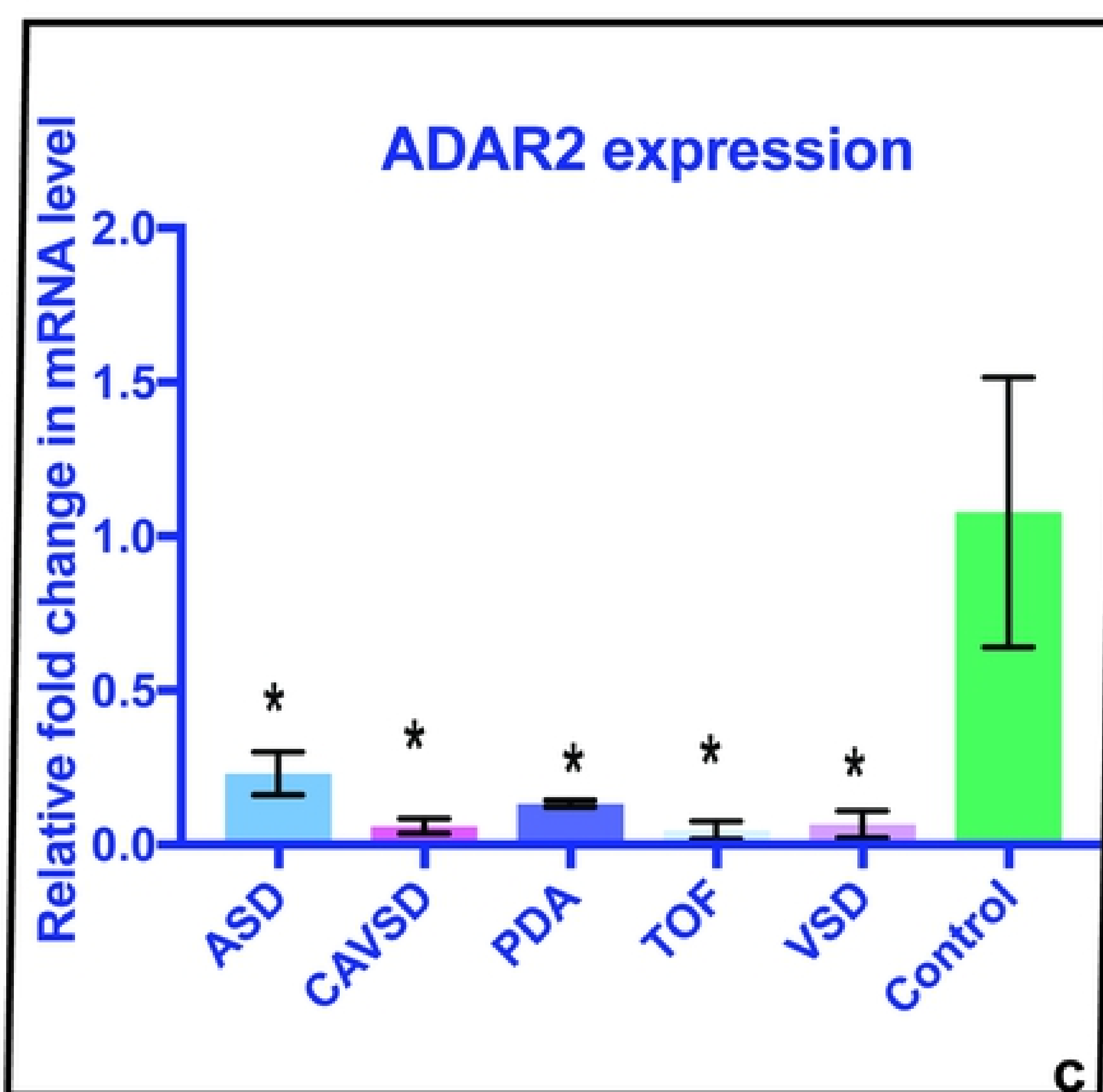
479

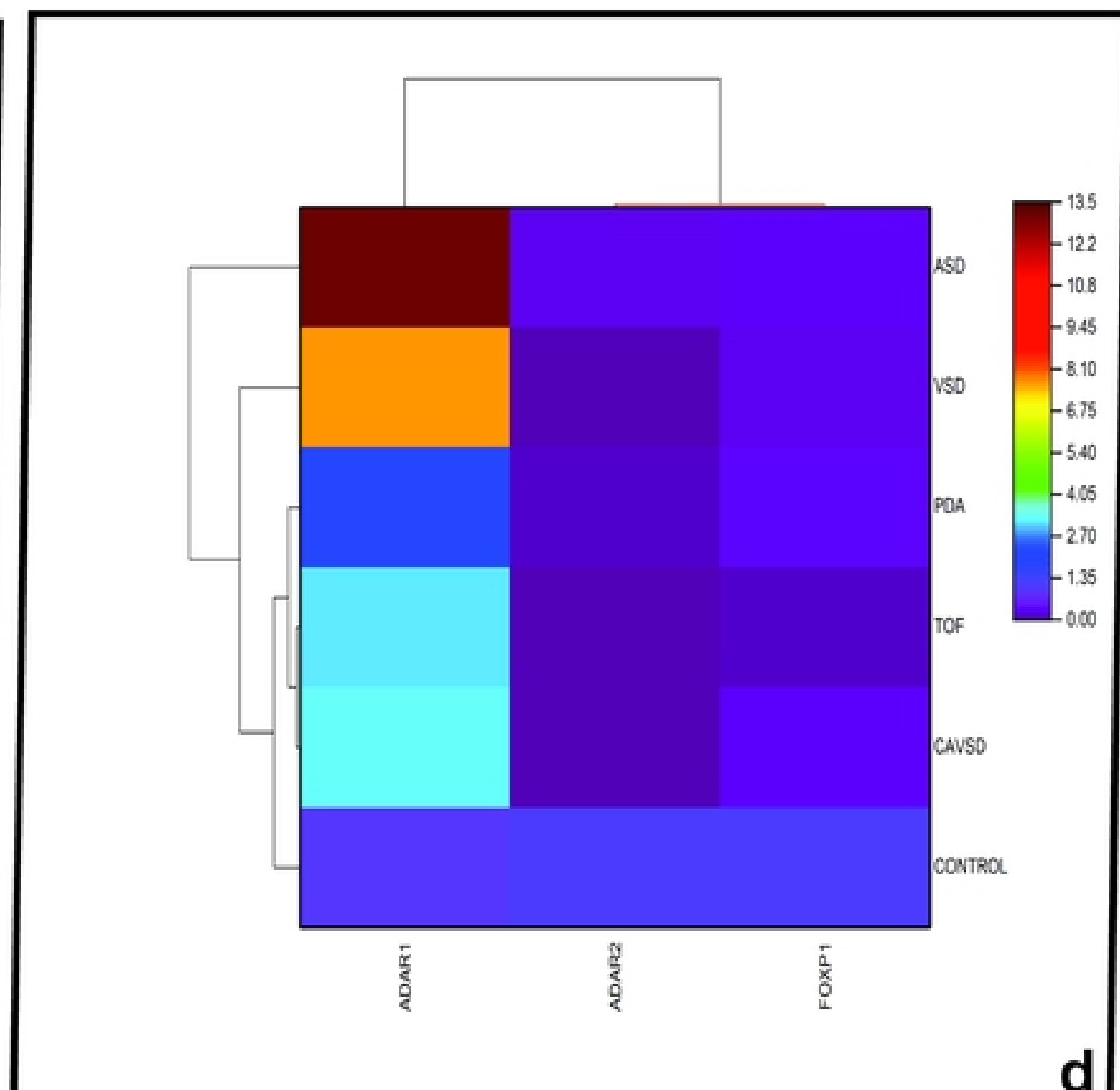

Relative fold change in mRNA level
15
10
5
2.0
1.5
1.0
0.5
0.0
*
*
*
ADAR1
ADAR2
FOXP1
CONTROL
AFFECTED

bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

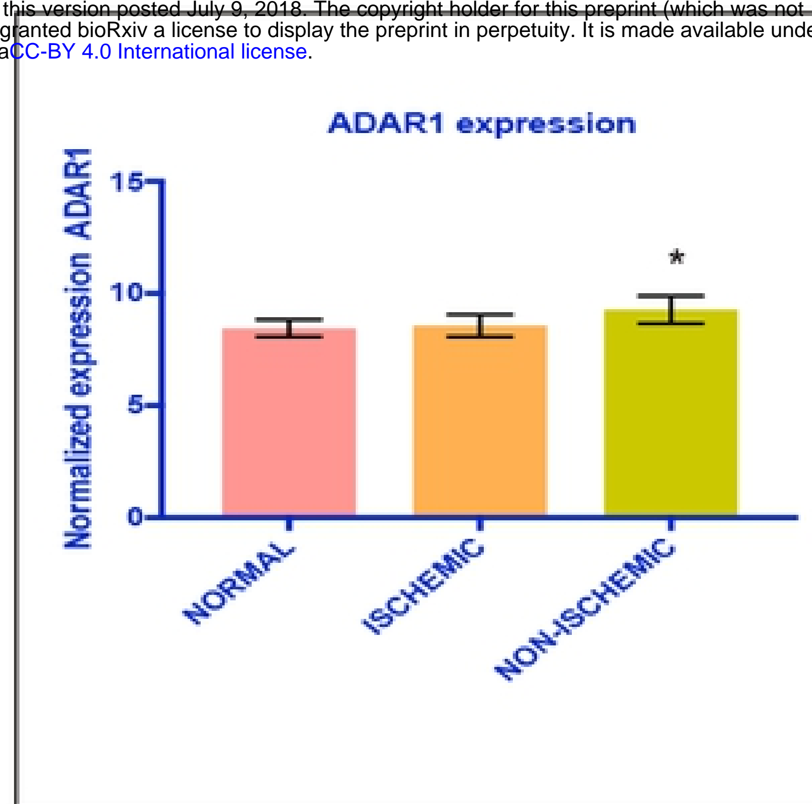


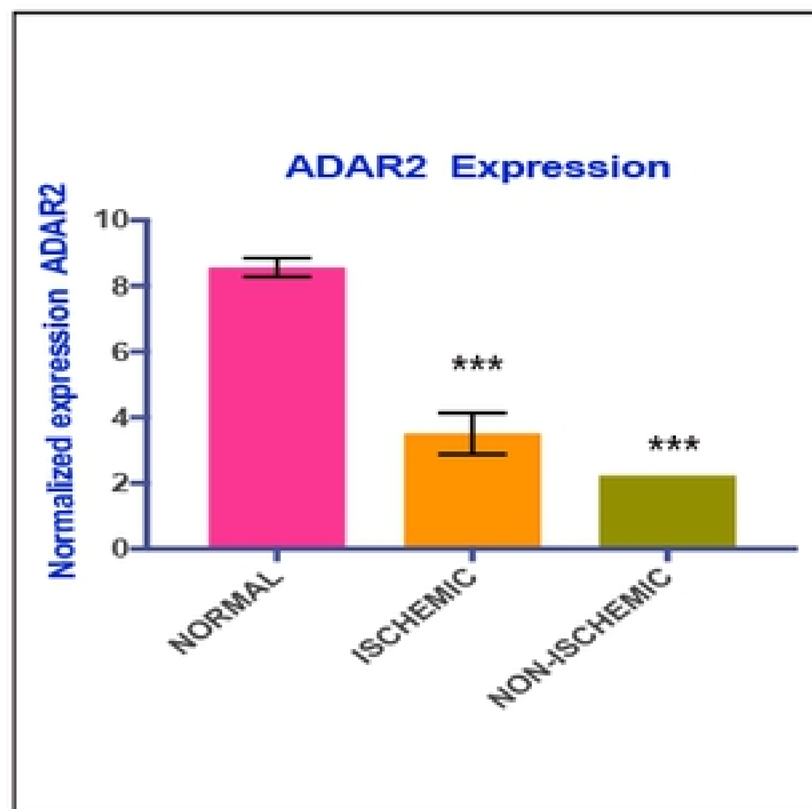






bioRxiv preprint doi: https://doi.org/10.1101/365288; this version posted July 9, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.





60 50

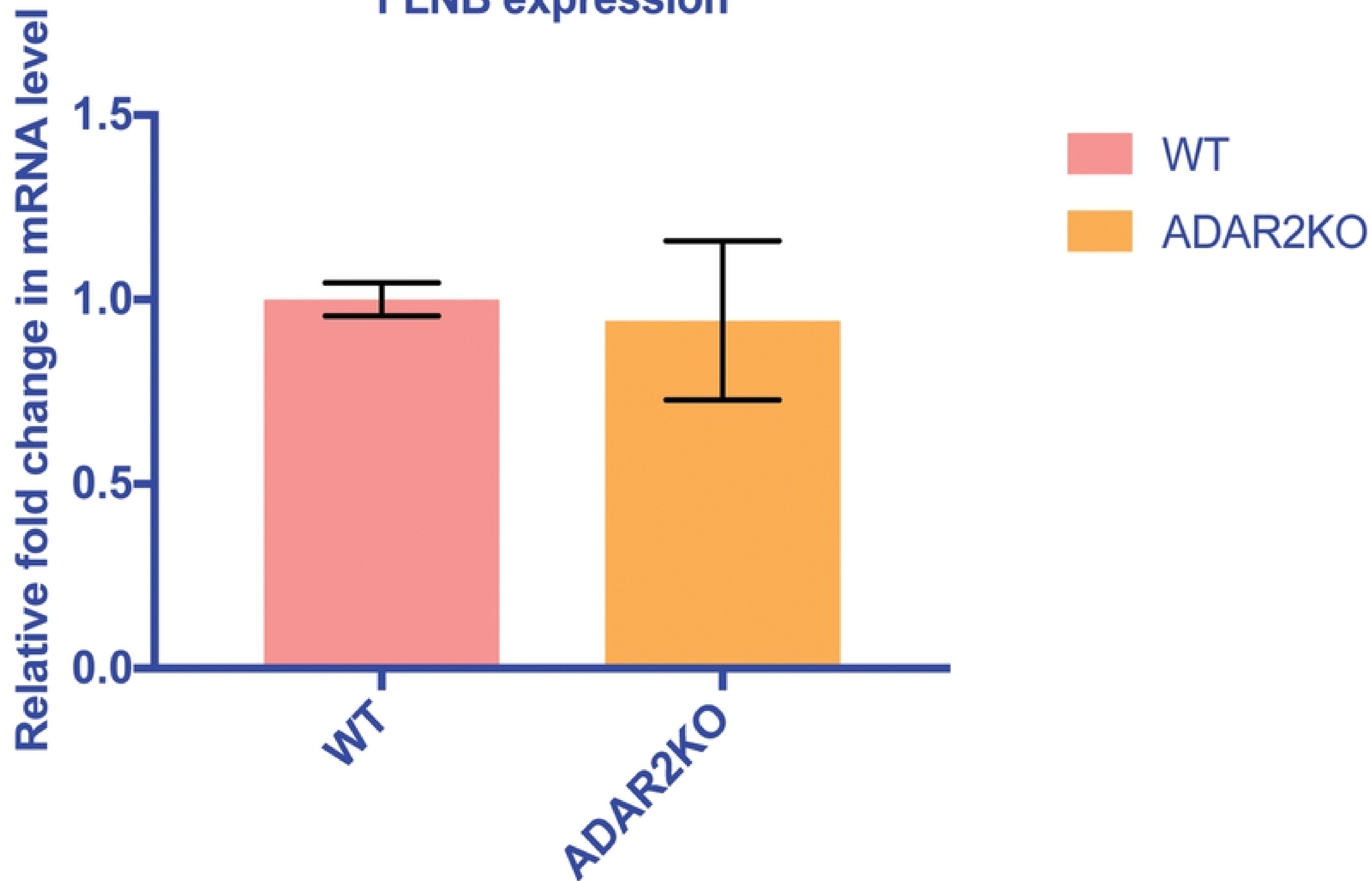
C A C T G T T C T G A G C C T T C A G G A A T

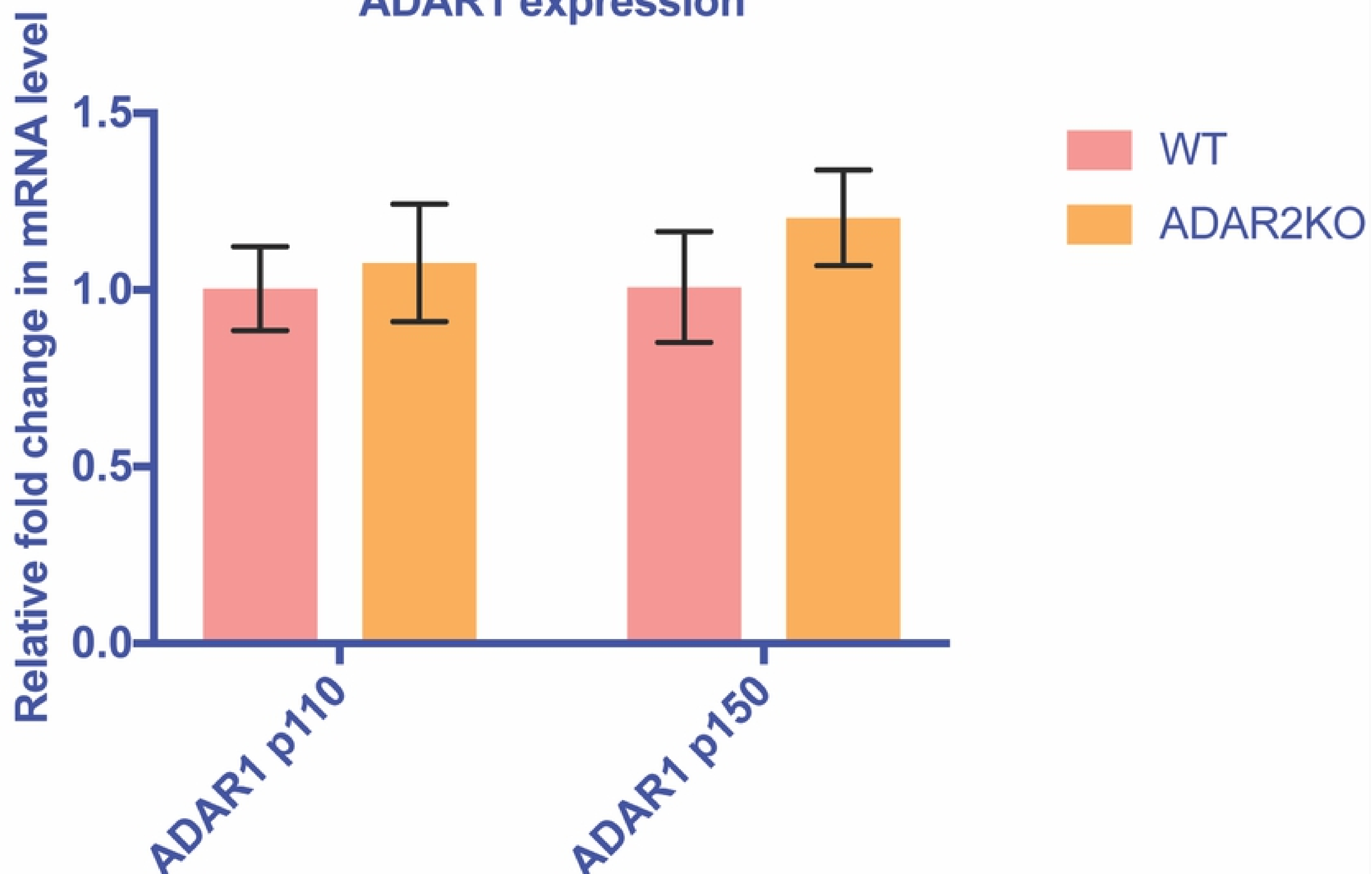
WT 42%

60 50

C A C T G T T C T G A G C C T T C A G G A A T

ADAR2KO 18%


FLNB expression
Relative fold change in mRNA level
1.5
1.0
0.5
0.0
WT
ADAR2KO
WT
ADAR2KO


ADAR1 expression
Relative fold change in mRNA level
1.5
1.0
0.5
0.0
WT
ADAR2KO
ADAR1 p110
ADAR1 p150