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Next Generation Sequencing of RNA reveals novel targets of Resveratrol with possible implications for Canavan disease

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Abstract:

Resveratrol (RSV) is a small compound first identified as an activator of sirtuin 1 (SIRT1), a key factor in mediating the effects of caloric restriction. Since then, RSV received great attention for its widespread beneficial effects on health and in connection to many diseases. RSV improves the metabolism and the mitochondrial function, and more recently it was shown to restore fatty acid β-oxidation (FAO) capacities in patient fibroblasts harboring mutations with residual enzyme activity. Many of RSV’s beneficial effects are mediated by the transcriptional coactivator PGC-1α, a direct target of SIRT1 and a master regulator of the mitochondrial fatty acid oxidation. Despite numerous studies RSV’s mechanism of action is still not completely elucidated. Our aim was to investigate the effects of RSV on gene regulation on a wide scale, possibly to detect novel genes whose up-regulation by RSV may be of interest with respect to disease treatment. We performed Next Generation Sequencing of RNA on normal fibroblasts treated with RSV. To investigate whether the effects of RSV are mediated through SIRT1 we expanded the analysis to include SIRT1-knockdown fibroblasts. We identified the aspartoacylase (ASPA) gene, mutated in Canavan disease, to be strongly up-regulated by RSV in several cell lines, including Canavan disease fibroblasts. We further link RSV to the up-regulation of other genes involved in myelination including the glial specific transcription factors POU3F1, POU3F2, and myelin basic protein (MBP). We also observe a strong up-regulation by RSV of the riboflavin transporter gene SLC52a1. Mutations in SLC52a1 cause transient multiple acyl-CoA dehydrogenase deficiency (MADD). Our analysis of alternative splicing identified novel metabolically important genes affected by RSV, among which is particularly interesting the α subunit of the stimulatory G protein (Gsa), which regulates the cellular levels of cAMP through adenylyl cyclase.

We conclude that in fibroblasts RSV stimulates the PGC-1α and p53 pathways, and up-regulates genes affecting the glucose metabolism, mitochondrial β-oxidation, and mitochondrial biogenesis. We further confirm that RSV might be a relevant treatment in the correction of FAO deficiencies and we suggest that treatment in other metabolic disorders including Canavan disease and MADD might be also beneficial.

Keywords: RNA-seq; resveratrol; ASPA; Canavan disease; Sirtuin 1; alternative splicing; p53; Fatty acid oxidation.
Abbreviations:

**ACADM**: acyl-CoA dehydrogenase medium chain; **ACADVL**: acyl-CoA dehydrogenase very long chain; **AMP**: adenosine monophosphate; **ATP**: adenosine triphosphate; **AMPK**: AMP-activated protein kinase; **ASPA**: aspartoacylase; **BBC3**: BCL2 binding component 3; **CDKN1A**: cyclin-dependent kinase inhibitor 1A; **CNS**: central nervous system; **COL16a1**: collagen XIV; **CPT1a**: carnitine palmitoyltransferase 1A; **CPT2**: carnitine palmitoyltransferase 2; **DMSO**: dimethyl sulfoxide; **E2F1**: E2F transcription factor 1; **FAO**: fatty acid β-oxidation; **FBLN2**: fibulin 2; **FDR**: false discovery rate; **FOXO**: forkhead box protein O; **GADD45A**: growth arrest and DNA-damage-inducible alpha; **GNAS**: guanine nucleotide binding protein, alpha stimulating; **Gsα**: α subunit of the stimulatory G protein; **INSR**: insulin receptor; **KEGG**: Kyoto Encyclopedia Genes and Genomes; **LKB1**: liver kinase B1; **MADD**: multiple acyl-CoA dehydrogenase deficiency; **MBP**: myelin basic protein; **MCAD**: medium-chain acyl-CoA dehydrogenase; **MDM2**: murine double minute 2; **NAA**: N-acetyl-L-aspartic acid; **NAD+**: nicotinamide adenine dinucleotide; **NESP55**: neuroendocrine secretory protein 55; **PDC**: pyruvate dehydrogenase complex; **PDE**: phosphodiesterase; **PDK4**: pyruvate dehydrogenase kinase isozyme 4; **PGC-1α**: peroxisome proliferator-activated receptor-γ co-activator 1α; **p53**: protein 53; **RNAseq**: RNA sequencing; **RPL13a**: ribosomal protein L13a; **RRAD**: ras-related associated with diabetes; **RSV**: resveratrol; **SIRT1**: sirtuin 1; **SMN**: survival motor neuron; **SRSF3**: serine/arginine-rich splicing factor 3; **TBP**: TATA-binding protein; **TCA**: tricarboxylic acid; **TFAM**: mitochondrial transcription factor A; **TFB1M**: mitochondrial transcription factor B1; **VLCAD**: very long chain acyl-CoA dehydrogenase, XLαs: extra-large Gsα.
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1. Introduction

Resveratrol (RSV; 3, 5, 4’-trihydroxy-trans-stilbene) is a small polyphenol found in grapes, berries, and nuts that received great attention due to its therapeutic potential in many diseases, including cardiovascular and neurodegenerative disorders, diabetes, and cancer. RSV is known to extend the lifespan of many organisms, delay the onset of age-related diseases, and have general beneficial effects on human health [1]. RSV also has strong neuroprotective effects; for example, it ameliorates the Huntington disease in nematode models [2], it promotes clearance of amyloid β peptides, and it reduces oxidative stress and neuronal cell death in Alzheimer’s disease [3].

There are many mechanisms through which RSV acts: it enhances the metabolism, reduces oxidative stress and inflammation, acts as an anti-oxidant, affects nitric oxide synthesis, and it regulates DNA damage, cell cycle, apoptosis, and proliferation [1, 4, 5]. The current hypothesis is that RSV exerts many of its effects by acting as a caloric restriction mimetic [6]. Caloric restriction, or fasting, is known to improve health and extend lifespan across species. In obese humans RSV supplementation improved the mitochondrial function [7]; while in mice RSV protected against the negative effects of an obesogenic diet [8], and produced a global increase in the oxidative metabolism and aerobic capacity of the mitochondria [9]. RSV has been shown to have therapeutic potential for some mitochondrial fatty acid β-oxidation (FAO) and respiratory chain disorders [10].

FAO disorders are a large group of inherited metabolic diseases with clinical features often including hypoketotic hypoglycemia, arrhythmia, cardiomyopathy, progressive lipid storage myopathy, myoglobinuria, and rhabdomyolysis, reflecting the important role that fatty acids play as energy substrates in heart and skeletal muscle [11, 12]. Medium chain acyl-CoA dehydrogenase (MCAD) deficiency, carnitine palmitoyltransferase type 2 (CPT2) deficiency, and very long chain acyl-CoA dehydrogenase (VLCAD) deficiency are prevalent defects of FAO [12, 13]. RSV was shown to increase gene expression of the MCAD gene in mice [9], and to restore mitochondrial FAO capacity in patient cells harboring missense mutations in CPT2 and VLCAD [14, 15].

Canavan disease (OMIM#271900) is another inherited metabolic disorder, which is caused by defective catabolism of the N-acetyl-L-aspartic acid (NAA). Canavan disease is a fatal autosomal-recessive neurodegenerative disease characterized by compromised central nervous system (CNS) development, defects in myelination, astrocyte hypertrophy, and deformed mitochondria [16-18]. Patients exhibit elevated NAA levels due to the deficiency of the aspartoacylase (ASPA) enzyme, responsible for the degradation of NAA into acetate and aspartate [17, 19]. NAA is involved in
neuronal energy metabolism: it is synthesized in neuronal mitochondria and then it migrates to oligodendrocytes, where it is used as a source of acetate for myelin synthesis and other metabolic functions. Although disputed, it appears that the deficiency of acetate, which is necessary for correct myelin formation, is the etiological mechanism of Canavan disease, rather than the excess of NAA [16, 20]. Canavan disease is prevalent among the Ashkenazi Jewish population and the two most frequent mutations in the ASPA gene (c.854A>C; p.E285A and c.914C>A; p.A305E) both result in low, but detectable residual enzyme activity [21-23]. Even minor differences in residual enzyme activity may be important in determining disease severity [22, 23].

The mechanisms by which RSV exerts its effects have been extensively studied, and a great variety of molecular mechanisms have been identified [4]. The current hypothesis is that RSV exerts many of its beneficial effects on health via sirtuin 1 (SIRT1) [6]: RSV effects are consistent with the activation of SIRT1, and many of these effects can be abrogated by SIRT1 inhibition. However, there is considerable debate over SIRT1 as the mediator of RSV’s effects, as the initial in vitro finding that RSV activates SIRT1 through allosteric interaction [24] was later found to be highly dependent on the substrate used [25]. SIRT1 belongs to a conserved family of nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases, and is a key player in mediating effects of caloric restriction [26]. SIRT1 controls and affects many pathways and targets; its effects on the metabolism are enacted mainly through the modulation of the forkhead box protein O (FOXO) transcription factors, and the peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) [26, 27]. SIRT1 is also known to regulate the tumor suppressor protein p53 [28]. The fact that so many pathways are affected both by SIRT1 and RSV is consistent with the observed widespread beneficial effects of this signaling mechanism. The effects of RSV on the metabolism and longevity has usually been reported to occur through the activation of PGC-1α via SIRT1[4]. PGC-1α is a transcriptional coactivator and master regulator of mitochondrial function and oxidative metabolism [29]. However, the beneficial effects of SIRT1 activation overlap in many ways with pathways affected by AMP-activated protein kinase (AMPK) [30]. AMPK is a metabolic sensor triggered by elevated AMP/ATP ratio; when AMPK is activated, it switches on catabolic pathways that generate ATP, and switches off anabolic pathways that consume ATP [31]. It has been suggested that AMPK can activate SIRT1 by increasing NAD⁺, leading to the deacetylation of downstream SIRT1 targets and the activation of transcriptional programs as metabolic adaptation to cellular energy needs [27, 32]. AMPK also phosphorylates PGC-1α, which was suggested to be a prerequisite for activation by SIRT1 [27, 32, 33]. The precise molecular mechanisms of RSV action are not entirely clear.
RSV can activate both AMPK and SIRT1, and the two pathways are interconnected: AMPK increases NAD\(^+\) and activates SIRT1, while SIRT1 deacetylates the AMPK kinase LKB1, leading to activation of AMPK [34-39]. More recently it was proposed that RSV inhibits phosphodiesterases (PDEs), which generate an increase in the cellular pool of cyclic AMP (cAMP); this in turn activates AMPK, and subsequently SIRT1 [40]. Many of the effects of RSV on the metabolism are thus mediated through the stimulation of the AMPK/SIRT1/PGC-1\(\alpha\) axis, resulting in the transcriptional activation of genetic programs involved in mitochondrial biogenesis and oxidative metabolism [9, 34, 35, 40]. However, RSV has many other molecular targets, and many of its effects may be exerted through pathways independent from SIRT1.

In this study we investigated the general effects of RSV on gene expression and splicing by RNA-sequencing (RNAseq) analysis of cultured human fibroblasts. In particular, we aimed to identify new metabolic disorders that might benefit from treatment with RSV. We further address the question of whether RSV exerts specific actions through SIRT1, by analyzing the gene expression in SIRT1-deficient cells by siRNA-mediated knockdown.

2. Materials and Methods

2.1 Cell culture and treatments

All cell lines were cultured at 37 °C, in 5% CO\(_2\), in RPMI1640 medium with 10% fetal calf serum, 1% penicillin/streptomycin, and 1% L-glutamine. We used three separate human fibroblast cell lines from healthy controls, and two Canavan patient fibroblast cell lines: one carrying the p.A305E mutation (CD01) obtained from M Angeles Ruiz, Pediatrics, Hospital Son Espases, Mallorca, Spain and Judit Garcia-Villoria, Div. Inborn Errors of Metabolism, BGM, Hospital Clinic, IDIBAPS, Barcelona and CIBERER, Madrid, Spain (informed consent was obtained); and one carrying the most common Jewish mutation resulting in p.E285A (GM04268 obtained from the Coriell Institute for Medical research, US). Additionally HepG2 liver cells and SH-SY5Y human neuroblastoma cells purchased from ATCC (LGC Standards GmbH, Germany) were used. Purified RSV (R5010; Sigma-Aldrich, MO, US) was used at 75µM for 48h for the treatments, unless otherwise stated, and it was dissolved in DMSO and diluted in RPMI1640. Knockdown of SIRT1 was achieved by transfection with SIRT1 siRNA (LQ-003540-00; Dharmacon, Boulder, US). Knock-down was
assessed by western blot probed with anti-SIRT1 antibody (sc-15404, Santa Cruz, CA, US) and anti-β-actin antibody (ab8229, abcam, Cambridge, UK).

2.2 Sample preparation and RNA-sequencing

Total RNA from two fibroblast SIRT1 knockdown and two control cell lines was extracted with Isol-RNA lysis agent (5PRIME GmbH, Hamburg, DE). Sample preparation and sequencing was performed essentially as described in Doktor et al. 2017 [41]. RNA purity, integrity, and concentration were determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., CA, US) and only RNA with a RIN value of 8.0 or higher and a 28s/18s ratio above 1.8 was used for sample preparation. The libraries were prepared according to Illumina TruSeq RNA Sample Prep Kit protocols, quantified with KaPa Library quantification Kits (Cat KK4824; KaPa Biosystems, Roche, CH) and a final concentration of 15 pM denatured libraries were used for pair-end 100bp sequencing on an Illumina HiSeq1500 (Illumina, CA, US).

2.3 Software analysis

RNAseq reads were aligned to the human genome Ensembl v.89 (hg38) using STAR v.253a [42] and DESeq [43] was used to identify differentially expressed genes. Genes with adjusted p-values lower than 0.1 were considered significantly changed. Differential splicing analysis was performed using DEXseq [44]. An FDR cut-off at 10% was set in order to identify alternatively spliced exons. KEGG pathways analysis were performed using ClusterProfiler [45]. Pathways with adjusted p-values below 0.05 were considered significant.

2.4 Sequencing data

Sequencing data are available from the ArrayExpress under accession number E-MTAB-7360.

2.5 qRT-PCR, RT-PCR, and splicing analysis

0.5-1 μg total RNA was used for reverse-transcription using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, CA, US) according to manufacturer instructions but with an extended synthesis at 42°C for 120 min. Subsequently, the cDNA was added 30 μl DEPC-water and cDNA concentrations were measured by absorbance readings at 260, 280 and 230 nm (NanoDrop™1000 Spectrophotometer; Thermo Scientific, CA, US). RT-PCR was performed using Tempase Hotstart Mastermix (Ampliqon, Odense, DK) and analysed by agarose gel electrophoresis and/or quantified
on a Fragment Analyzer™ (Advanced Analytical Technologies, IA, US). qRT-PCR was performed on Roche LightCycler 480 with SYBR-green premix FastStart Essential DNA Green Master (Roche Diagnostics, CH) according to manufacturer instructions. Each qRT-PCR assay included a standard curve of 7 serial dilutions of a cDNA mix of all the samples (250 to 0.016 ng) and a no-template control. Whenever possible, the primers were designed to span at least once over adjacent exons. When primers did not span over multiple exons, an additional control was included in the qRT-PCR analysis where equal volumes of RNA were subjected to qRT-PCR to test for genomic DNA contamination. For the complete list of primers see supplementary data. PCR efficiencies (\(=10(-1/slope) -1\)) were >70% and \(r^2 = 0.96\) or higher. The specificity of each amplification was analyzed by melting curve analysis. Quantification cycle (Cq) was determined for each sample and the comparative method was used to detect relative gene expression ratio normalized to the average of two reference genes (\(TBP\) and \(RPL13a\)). Significance was ascertained by the two-tailed Student’s t-test.

3. Results

Cultured fibroblasts are often the model choice for the study of genetic disorders, including inherited disorders of metabolism. Fibroblast cells are relatively easy to obtain and can usually be used as model for investigating the molecular pathology of mutations. We treated two different fibroblast cell lines with RSV and performed RNAseq analysis to investigate the global changes that RSV induced on gene expression. SIRT1 mediates many of the RSV’s effects, although the precise mechanism is unclear yet, and RSV acts also through SIRT1-independent mechanisms. To detect the changes induced by RSV that are dependent on SIRT1, we performed a SIRT1 knockdown. We confirmed the knock-down by qRT-PCR and western blot (figure 1) and, interestingly, we observed that RSV increased SIRT1 transcription and protein level, indicating that RSV activates SIRT1 transcriptionally.

Our RNAseq analysis showed that RSV up-regulated 205 genes and down-regulated 140 genes. In the same analysis on RSV-treated cells where SIRT1 was knocked down, we observed fewer genes regulated in response to RSV: 111 up-regulated and 168 down-regulated, consistent with the fact that many effects of RSV are SIRT1-dependent. However, 65.2% of the genes affected by RSV in the control group are still affected by RSV when SIRT1 is knocked down, arguing to the fact that
RSV also affects the expression of many genes through SIRT1-independent pathways. 19.4% of gene changes in the SIRT1 knockdown group are exclusive to this group, which may be a consequence of SIRT1 knockdown by itself.

Figure 1. Validation of SIRT1 knock-down

qRT-PCR (A) and western blot (B) analysis of SIRT1 mRNA and protein levels respectively, confirming the SIRT1 knockdown. The fibroblasts were treated either with scrambled siRNA (siNT), siRNA targeted at SIRT1 (siSIRT1) in addition to 75 μM RSV treatment. The relative expression is normalized to the average of two control genes: RPL13a and TBP. The error bars show standard deviations. * indicates significant p-values when p < 0.05 as assayed by the t-test. (C) Venn diagram of the overlap of significantly changed genes between RSV-treated control fibroblasts (RSV) and SIRT1 knockdown (RSV + SIRT1-KD) fibroblasts.
To validate our RNAseq data, we selected a total of 22 genes and analyzed them by quantitative RT-PCR (qRT-PCR) (supplementary data, figure A.3). We chose known and novel targets of RSV from different areas including metabolic, cancer-related, and neuronal genes; and we included in the validation analysis an additional control fibroblast cell line. Variations in gene expression between different fibroblast cell lines are not unusual, as different fibroblast cell lines derive from different individuals, thus they contain different and unique genotypes. We decided to use three different cell lines as we reasoned that this experimental model would be more representative of the real variation that RSV treatment can elicit. For a direct comparison between RNAseq and qRT-PCR we compared the log2fold increase for each tested gene (supplementary data, figure A.2). Although changes in gene expression varied in magnitude according to the two analyses, qRT-PCR analysis confirmed the trend in gene expression change for all except one gene (FOXO1). However, the change in gene expression for FOXO1 was not statistically significant in RNAseq nor qRT-PCR.

3.1 RSV up-regulates the PGC-1α pathway and FAO in normal human fibroblasts

Activation of the mitochondrial FAO represents the physiological response to energy depletion, which occurs, for example, during fasting [46]. Previously, RSV was reported to increase mRNA and protein levels of two genes involved in fatty acid metabolism, CPT2 and ACADVL, in fibroblasts from patients with missense mutations that cause reduced enzymatic activity [14, 15]. Our aim was to investigate whether there are other metabolic genes up-regulated by RSV, with particular interest to genes where disease causing missense mutations result in some residual enzymatic activity. We first searched for known FAO genes affected by RSV. Our RNAseq analysis showed a robust increase of ACADVL expression and a rather modest increase in CPT1a, but no change for CPT2 and ACADM expression (Table A.4 in supplementary data).

We confirmed up-regulation of ACADVL in three normal fibroblast cell lines and we also observed a slight, albeit not always significant, increase of CPT1a expression (figure 2). Contrary to previous data, we did not always observe that these changes in gene expression were affected by SIRT1 knockdown. This was particularly clear for ACADVL and PDK4, where RSV mediated up-regulation was unaffected by SIRT1 knockdown. A possible explanation is that RSV elicits these changes independently of SIRT1 through the estrogen receptor, as previously observed [10].
Figure 2. Metabolism-related genes affected by RSV treatment in control human fibroblasts

qRT-PCR analysis of gene expression levels in three control fibroblast cell lines. Control (siNT) and SIRT1-knockdown (siSIRT1) cells were treated for 48h with 75μM RSV dissolved in DMSO, or DMSO alone. Relative expression values were normalized to two control genes (RPL13a and TBP), error bars are standard deviations.* indicates significant p-values when p < 0.05 as assayed by the t-test.

The highest up-regulation induced by RSV was observed for the Ras-related associated with diabetes (RRAD) gene (figure 2 and table A.4 in supplementary data). RRAD reduces insulin-stimulated glucose uptake [47] and is highly expressed in the heart. RRAD levels have been found to be decreased in failing human hearts and in association with cardiac hypertrophy, thus it was suggested for RRAD to have an important cardioprotective role [48]. Hypertrophic cardiomyopathy is a common feature in β-oxidation defects, like VLCAD deficiency, and RSV-induced increase in RRAD expression might be therefore beneficial in this context.
We also observed a strong up-regulation by RSV of the pyruvate dehydrogenase kinase, isozyme 4 (PDK4) gene (figure 2). PDK4 is an important regulator of glucose homeostasis. It is up-regulated during fasting and its transcription is regulated by PGC-1α [49, 50]. PDK4 inhibits the pyruvate dehydrogenase complex (PDC), which catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl CoA, thus PDK4 reduces the entry of glucose in the tricarboxylic acid (TCA) cycle. During fasting, when glucose levels are low and need to be preserved, PDC is inactivated by PDKs while FAO becomes the preferred pathway for production of acetyl CoA [51]. RSV, by increasing the transcription of PDK4, could thus generate a decrease in the production of ATP from glucose.

Consistent with the fact that RSV activation of PGC-1α and/or AMPK is known to be posttranslational, we did not observe an increased mRNA expression from genes encoding PGC-1α or AMPK regulatory subunits. However, we did observe an increased expression of the mitochondrial transcription factor A (TFAM) and mitochondrial transcription factor B1 (TFB1M) genes in the RNAseq data, although with not significant p-adjusted values (table A.4, supplementary data). Both TFAM and TFB1M are downstream factors in the PGC-1α-dependent cascade in mitochondrial biogenesis. TFAM expression decreases with age, and caloric restriction was found to reverse this decrease [52]. Overall, we observed that in normal fibroblasts the PGC-1α pathway is activated upon RSV treatment and that FAO may be stimulated by RSV through the up-regulation of several different β-oxidation genes.

3.2 RSV up-regulates SLC52a1 and ASPA, involved in MADD and Canavan disease, respectively.

One of the most strongly up-regulated genes in our data is the riboflavin transporter (SLC52a1) gene (figure 2). Mutations in the riboflavin transporter genes, including SLC52a1, are related to transient multiple acyl-CoA dehydrogenase deficiency (MADD) in newborns, which can be treated with riboflavin supplementation [53-55].

Our RNAseq data further revealed a strong increase in expression of ASPA, the gene mutated in Canavan disease. Because the most prevalent disease-causing mutation results in a low level of residual ASPA activity [21], an increase in ASPA expression levels could result in an increase in the enzyme activity to levels which may be of benefit to the patients. We confirmed by qRT-PCR that ASPA’s relative expression is increased upon RSV treatment in normal fibroblasts (figure 3A). When treating fibroblasts with increasing amounts of RSV (0-150 μM), we observed a dramatic up-
regulation of ASPA’s transcript levels in a dose-dependent manner, which peaks at 75 μM of RSV (figure 3B). We observed a similar up-regulation of ASPA in response to RSV treatment also in HepG2 cells (figure 3C). We also demonstrate that ASPA’s expression increases in a time-dependent manner with a steep increase after 10 hours of RSV stimulation, and by 48 hours the expression is increased by more than 12 fold (Figure 2D).

Figure 3. RSV increases the expression of ASPA

(A) ASPA’s relative expression is increased after treatment with RSV in fibroblasts. qRT-PCR analysis of ASPA’s relative gene expression levels in control (siNT) and SIRT1 (siSIRT1) knockdown fibroblasts after treatment with 75μM RSV for 48h. Fibroblasts were treated with RSV dissolved in DMSO, or DMSO alone. (B) RSV stimulates ASPA’s gene expression increase in a dose-dependent manner when assayed with qRT-PCR. Control fibroblasts (B) and HepG2 cells (C)
were treated with increasing doses (0 to 150 μM) of RSV for 48h. Representative agarose gel picture with RT-PCR bands are shown under the bar plot. ASPA’s gene expression increases in a time-dependent manner after treatment with RSV. (D) qRT-PCR analysis of ASPA’s relative expression on a time-curve stimulation with RSV. Control fibroblasts were treated with 75μM RSV; samples were collected and assayed at various time points until a maximum of 48h after start of stimulation. ASPA’s expression levels are normalized to RPL13A. Standard deviation is displayed as error bars. * indicates when p<0.05 in a two-tailed Student T test.

The strong RSV mediated up-regulation of ASPA expression could also be demonstrated in fibroblasts from two patients with Canavan disease (figure 4A), who harbor the p.A305E and p.E285A mutations. Moreover, RSV treatment up-regulates ASPA in SH-SY5Y neuroblastoma cells (figure 4B), indicating that the up-regulation occurs also in cells of neuronal origin.

Figure 4. RSV induces ASPA’s gene expression in Canavan disease patient cells

(A) qRT-PCR showing increased ASPA relative gene expression in two fibroblasts cell lines obtained from Canavan disease patients (CD01 and GM04268). Cells were treated with 75μM RSV in DMSO, or DMSO alone for 48h. (B) RSV increases ASPA’s level of expression in different cell types including Canavan disease patient’s cells: agarose gel picture of RT-PCR with ASPA specific
primers in fibroblasts, the GM04268 Canavan disease patient cell line, and SH-SY5Y cells treated either with 75μM RSV or DMSO alone for 48h.

ASPA is highly expressed in oligodendrocytes where it has an important role in myelination [20]. Therefore, we hypothesized that RSV might up-regulate also other important genes expressed in oligodendrocytes and/or play important roles in myelination. Our RNAseq results revealed that RSV increases the expression of two POU3 members: \textit{POU3F1} and \textit{POU3F2} (table A.4, supplementary data). The transcription factors \textit{POU3F1} and \textit{POU3F2} are important positive regulators of myelination during Schwann cell development, and they promote neurogenesis in the CNS [56-58]. qRT-PCR confirmed a strong increase in expression of both \textit{POU3F1} and \textit{POU3F2}, going from undetectable levels to a clear detection of the transcripts in human fibroblasts (figure 5A). This up-regulation occurred also when SIRT1 was knocked-down. \textit{POU3F1} and \textit{POU3F2} were also up-regulated by RSV in the GM04268 Canavan disease patient cell line (figure 5B). Interestingly, in fibroblasts treated by RSV we also detected a slight, but consistent increase in the relative expression levels of the myelin basic protein (MBP). MBP is a major constituent of myelin [59]. The RSV-mediated up-regulation of MBP was reduced when SIRT1 was knocked-down (figure 5A).
Figure 5. RSV up-regulates the POU3 transcription factors involved in myelination

*POU3F1, POU3F2, and MBP expression levels are increased in cells treated with RSV. (A) Expression levels of *POU3F1, POU3F2, and MBP* genes are assessed by qRT-PCR in fibroblasts treated with 75μM of RSV and DMSO, or DMSO alone, in control (siNT), and in SIRT1 knockdown (siSIRT1) cells. The values are normalized to the average of two control genes (*RPL13a* and *TBP*). Error bars are standard deviations. (* indicates significant p-values when p <
0.05 as assayed by the t-test. B) Representative agarose gel electrophoresis of RT-PCR with primers specific for POU3F1 and POU3F2 in control and RSV-treated (75μM, 48h) fibroblasts, and cells obtained from Canavan disease patients. HPRT is the control gene.

Taken together, these results show that RSV up-regulates ASPA expression in a SIRT1-independent manner in several different cell lines, including two Canavan disease cell lines. RSV further up-regulates other genes, POU3F1, POU3F2, and MBP, which are important for myelination. However, MBP is up-regulated in a SIRT1-dependent manner while ASPA, POU3F1, and POU3F2 are not. This indicates that there are different pathways involved in the RSV mediated up-regulation of genes involved in myelination.

3.3 RSV up-regulates genes involved in the p53 pathway

To identify other pathways that are affected by RSV treatment we performed a cluster analysis (KEGG pathways with ClusterProfiler). Among the most significantly up-regulated pathways by RSV were the p53 signaling pathway, the cell cycle, cellular senescence, and apoptosis pathways (table 1, the complete list is in the supplementary data, table A.3). RSV has been previously described to up-regulate the tumor-suppressor protein p53 [60]. p53 is activated upon cellular stress, e.g. DNA damage, and it initiates transcriptional programs that induce DNA repair, cell cycle arrest, and apoptosis [61]. p53 also mediates part of its anti-cancer activity through the regulation of cellular energy metabolism and oxidative stress; and it is able to modulate glycolysis, oxidative phosphorylation, fatty acid synthesis, and oxidation [62]. In our dataset the p53 pathway is still up-regulated when SIRT1 is knocked-down, indicating that the regulation can occur independently from SIRT1.

Table 1. KEGG pathways analysis of RNAseq data performed on RSV-treated fibroblasts

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<td>RSV + SIRT1 KD</td>
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<td>1</td>
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<td></td>
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<td>2</td>
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<tr>
<td>3</td>
<td>Complement and coagulation cascades</td>
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<td>08/115</td>
<td>0,0095</td>
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Top 20 most significant pathways up- and down-regulated by RSV. The table summarizes which pathway are affected by RSV in control and SIRT1 knock-down (SIRT1 KD) fibroblasts: if the regulation is abolished upon SIRT1 knockdown it means that RSV regulates the pathway through a SIRT1-dependent mechanisms.
Consistent with an activation of p53 through posttranslational modifications, we did not observe an effect of RSV on the p53 transcript amounts. We confirmed by qRT-PCR the up-regulation of several known factors in the p53 network: the E2F transcription factor 1 (E2F1), cyclin-dependent kinase inhibitor 1A (CDKN1A) or p21, BCL2 binding component 3 (BBC3), and the growth arrest and DNA-damage-inducible alpha (GADD45A) genes (figure 6). We detected a clear SIRT1-dependent up-regulation of the negative p53-regulator, murine double minute 2 (MDM2) (figure 6).

**Figure 6. Cancer-related genes affected by RSV treatment in fibroblasts**

qRT-PCR validation of cancer-related genes where expression is affected by RSV treatment in three different fibroblast cell lines. The histograms show the relative gene expression values normalized to the average of TBP and RPL13a, in control (siNT), and SIRT1 knockdown (siSIRT1) fibroblast stimulated with 75μM RSV and DMSO, or DMSO alone for 48h. No bars are plotted when the transcript levels were under the level of detection. Results are shown as mean values; the error bars are standard deviations. Significant p-values were calculated using the t test and * indicates p<0.05.

3.4 RSV affects the splicing of two extracellular matrix proteins and the GNAS locus
Splicing regulation and changes in the expression of splicing factors has been found to be associated with longevity in humans [63], and caloric restriction was shown to oppose the changes in alternative splicing that occur with ageing in nematodes [64]. Therefore we analyzed if RSV as a caloric restriction mimetic affected alternative splicing. The effect of RSV on alternative splicing is limited, but it has been reported to affect a few genes including: the insulin receptor gene (INSR), the survival motor neuron gene 2 (SMN2), and the serine/arginine-rich splicing factor 3 (SRSF3) [65-67].

Our analysis of RSV induced alternative splicing by differential exon splicing analysis (DEXseq) identified a total of 77 genes (supplementary data, Table A.6). Of these, eight genes showed alternative splicing in exons other than the first and the last (which are usually alternative 5' and 3' untranslated regions). We validated that RSV alters the inclusion of alternative exons in four of these genes (figure 7). Despite not being identified in the DEXseq analysis, we included SMN2 in the analysis, because its splicing was previously described to be affected by RSV and SMN1/SMN2 are not detected by the DEXseq analysis due to the high similarity between SMN2 and SMN1 gene sequences [68]. In particular, we observed a decrease in the inclusion of exon 41 in the Collagen XIV (COL16a1) gene upon RSV treatment, and an increase in the inclusion of exon 4 in SRSF3 (although only in two of three cell lines), exon 9 in Fibulin 2 (FBLN2), and exon 3 in the GNAS locus. The GNAS locus codes for five different transcripts: α-subunit of the heterotrimeric stimulatory G protein (Gsα), extra-large Gsα (XLαs), neuroendocrine secretory protein 55 (NESP55), and two non-coding transcript called A/B and GNAS-AS1. Gsα, XLαs, NESP55, and A/B originate from different promoters but share exon 2-13 [69]. Therefore, RSV might affect all four transcripts from the GNAS locus. The observed changes are independent from the SIRT1 pathway, because none of them are affected by SIRT1 knockdown.
Figure 7. RSV induces alternative splicing independently of SIRT1

Splicing analysis after RSV treatment in control (siNT) and SIRT1-knockdown (siSIRT1) fibroblasts. (A) Agarose gel electrophoresis of RT-PCR analysis performed on three fibroblast cell lines. RSV induces alternative splicing of several genes but the splicing pattern is unaffected by SIRT1 knockdown (B) Quantification of the RT-PCR bands in control and RSV-treated cells. The intensity of the bands was quantified as percentage over total intensity in the lane with a Fragments Analyzer™. In the histograms are plotted mean values from two RT-PCR experiments and the error bars are standard deviations. * indicates significant p-values when p < 0.05 as assayed by the t-test.

4. Discussion

Resveratrol is known to increase the energy metabolism and to stimulate many mitochondrial pathways including mitochondrial β-oxidation. Because RSV was found to increase FAO capacities in patient fibroblasts harboring VLCAD and CPT2 missense mutations that result in residual enzyme activity, it has been proposed as a promising molecule for pharmacological treatment of
VLCAD and CPT2 deficiencies [14]. However, although RSV may appear as a very appealing treatment when focusing on the positive effect on a single gene, like ACADVL, it is important also to consider that RSV treatment will simultaneously affect other genes, and that this may have both counterproductive and/or synergistic effects. In order to reveal other RSV target genes, we performed RNAseq analysis of fibroblasts stimulated with RSV in combination with or without siRNA-mediated knockdown of SIRT1. Many effects of RSV are enacted through the activation of SIRT1, initially thought to occur through direct binding [24], but this finding was later brought into question as it was shown to be highly substrate-dependent [25]. The exact mechanism of how RSV activates SIRT1 is not yet clear, and it is disputed whether it occurs indirectly through phosphorylation by AMPK or another indirect mechanism. Recently, RSV was shown to increase SIRT1 expression and activity in human chondrosarcoma tumor cells [70]. In agreement with these findings, we show that in normal human fibroblasts RSV triggers the transcriptional activation of SIRT1, and increases SIRT1 protein levels. Whether the increase in the abundance of the SIRT1 protein is prior, secondary, or concomitant to an increase in SIRT1 protein activity remains to be determined.

As expected we observed RSV mediated up-regulation of metabolic and mitochondrial genes under the transcriptional control of PGC-1α, and we also identified novel targets of RSV in relation to metabolic disease.

We confirm transcriptional up-regulation of ACADVL in normal human fibroblasts and we show that RSV also produces a strong up-regulation of RRAD. RRAD is known to inhibit insulin-stimulated cellular uptake of glucose and is a direct transcriptional target of p53 [71, 72]. RRAD is highly expressed in heart, lung, and skeletal muscle, and it is highly expressed early after skeletal muscle injury and muscle regeneration [73]. It was suggested for RRAD to have an important cardioprotective role as it can inhibit features of cardiac hypertrophy in cultured cells [48]. Hypertrophic cardiomyopathy is a common clinical feature in VLCAD deficiency and up-regulation of RRAD could therefore have a potentially beneficial effect. However, mice with RRAD overexpression in muscle develop glucose intolerance due to increased insulin resistance [74] and it is unclear how such an effect would affect patients with VLCAD deficiency when treated with RSV.

We identified also the ASPA and the SLC52a1 genes, involved in Canavan disease and MADD respectively, to be up-regulated transcriptionally by RSV. Canavan disease is a fatal disorder with
compromised myelination as one of the major features due to impaired activity of the ASPA enzyme [75]. To date, more than 100 ASPA mutations have been described, and the most prevalent result in low levels of residual activity of the enzyme (http://www.lovd.nl/ASPA) [21-23, 76]. In a recent study, Mendes et al. showed that there is a correlation between ASPA residual activity and disease severity in Canavan disease. Notably, there are only very small differences (2.5% vs 5.5% of wild-type) in residual activity between mutant proteins associated with milder and severe phenotypes, suggesting that even a small increase in the enzyme’s activity can have an impact on the quality of life of patients [23]. Currently, there is no pharmacological treatment for Canavan disease, although gene therapy targeted at delivering functional ASPA to oligodendrocytes reverted the pathology in a Canavan disease mouse model [77]. We describe a strong transcriptional up-regulation of the ASPA gene upon RSV treatment in several cell lines, including two Canavan disease patient cells, and in a dose- and time-dependent manner. We show that RSV also up-regulates other genes important for myelination (POU3F1, POU3F2, and MBP), which might suggest a general beneficial effect of RSV on myelination. RSV might promote the increase in ASPA’s transcription through direct binding of POU3F1 and POU3F2 transcription factors to the ASPA promoter. ASPA is involved in correct myelin formation: in oligodendrocytes it provides the acetate necessary for myelin synthesis [17]. POU3F1 and POU3F2 initiate transcription programs involved in the development and function of the nervous system. POU3F1 is highly expressed in embryonic stem cells, oligodendrocyte precursors, and Schwan cells, and both factors are important for myelination of peripheral nerves [58, 59]. Another possibility is that RSV regulates ASPA through a methylation mechanism. RSV has previously been shown to change the methylation pattern of specific genes [78, 79]. Moreover, distinct patterns of CpG methylation are related to ageing, and in particular, the CpG methylation status of ASPA appears to be a hallmark of ageing. Notably, human chronological age can be predicted with 5 years accuracy based on the epigenetic signature of only three genes among which is ASPA [80]. We conclude that RSV may have potential as treatment in Canavan disease through the up-regulation of ASPA, but this should be further investigated.

Our RNAseq data shows that RSV up-regulates the p53 pathway, which is in agreement with previous work in cancer cell lines [81-83]. The up-regulation of the p53 pathway that we observe occurs through a mechanism that does not require SIRT1. p53 is a critical tumor-suppressor protein, which blocks tumor development by three different pathways that promote cell cycle arrest, DNA repair, and apoptosis [61]. Our data show RSV mediated transcriptional up-regulation of many of
the p53 targets involved in all three pathways. Some examples include: \textit{GADD45A} (DNA repair), \textit{BBC3} (apoptosis), and \textit{CDKN1A} (cell cycle). In contrast to our finding of up-regulation of the p53 pathway, we also observed that expression of the p53 regulator, \textit{MDM2}, was increased by RSV, but in a SIRT1-dependent way. The significance of this remains to be elucidated, but it may suggest that alternative RSV signaling pathways mediate the up-regulation of p53 stimulated genes and the negative p53 regulator MDM2. More recently, a new interesting function of p53 was discovered, namely that p53 can suppress oncogenesis, by opposing key metabolic changes that occur in cancer cells. Many cancer cells have an altered metabolism in order to sustain rapid growth and cell division. This alteration is coined the Warburg effect and represents a shift from oxidative phosphorylation to glycolysis for an increased ATP production \cite{84}. p53 reverses the Warburg effect by antagonizing the metabolic adaptations that occurs in cancer cells \cite{62,85}. We show that RSV transcriptionally regulates genes that participate in this shift. For example, it up-regulates the expression of \textit{E2F1}, which besides being important for the regulation of the cell cycle and apoptosis, also regulates metabolism by mediating the switch from the oxidative to the glycolytic pathways \cite{86}. In line with this we show that, \textit{RRAD}, which is repressed in a number of cancers leading to increased uptake of glucose \cite{71,72,87-90}, is strongly up-regulated by RSV. We conclude that in cultured human fibroblasts RSV has a SIRT1-independent effect on important factors that affect all pathways influenced by p53, namely cell cycle arrest, DNA repair, apoptosis, and the Warburg effect.

Changes in alternative splicing is a hallmark of ageing, and RSV has been previously shown to affect splicing. Our global analysis of splicing changes identified 77 genes where alternative splicing is affected by RSV. We confirm previously reported changes in alternative splicing and report four novel targets with RSV mediated altered splicing pattern. Splicing of an artificial \textit{SRSF3} minigene was previously described to be affected by RSV \cite{66}. Here, we confirm that RSV increases inclusion of exon 4 in endogenous \textit{SRSF3} transcripts in human fibroblasts. Exon 4 inclusion in \textit{SRSF3} mRNA is a mechanism of autoregulation where the generated transcript is degraded through non-sense mediated decay \cite{91}. It could be speculated that this change in the splicing of a well-known splicing regulatory protein, in turn affects other alternative splicing events, like the ones we observe in this study.

Interestingly, RSV increases the inclusion of exon 3 of \textit{GNAS}. The best characterized product of \textit{GNAS} is the transcript encoding the Gsα protein, which is essential in signal transduction. Gsα
activates many effector proteins, including adenylyl cyclase [92]. Adenylyl cyclase catalyzes the synthesis of cAMP from ATP. This, in turn, can activate AMPK [40], an important player in the AMPK/SIRT1/PGC-1α axis stimulated by RSV (Figure 8). Interestingly, Gsα function is also related to energy expenditure and Gsα knockout mice develop obesity, glucose intolerance, and insulin resistance [93]. Gsα has at least five splicing variants, and the long and short isoforms are defined by the inclusion or exclusion of exon 3. Although differences in the Gsα activity have been reported for the two isoforms, their biological significance is not yet clear [89]. Here we show that RSV induces an increase in the inclusion of exon 3 in the Gsα transcript in fibroblasts, thus it promotes the longer isoform. RSV is also known to increase cAMP levels by inhibiting PDEs, and consequently, to stimulate the AMPK/SIRT1/PGC-1α pathway [38]. A switch in the splicing isoforms of Gsα might be the result of a feedback mechanism of the initial PDE inhibition and activation of metabolic programs (figure 8).

**Figure 8. Proposed model of RSV’s mechanism of action**

RSV is known to affect the mitochondrial function by inhibiting PDEs and thus change the ATP/AMP ratio. RSV could affect the pool of cAMP also by modulating the activity of adenylyl
cyclase, possibly through a feedback mechanism that results in the induction of the long isoform of the alpha subunit of the G protein – Gsα. Gsα couples seven-transmembrane receptors to adenylyl cyclase. AMPK activation results in phosphorylation of the transcription factor PGC-1α, and increased cellular levels of NAD⁺. Increased NAD⁺ activates SIRT1, which then deacetylates and activates PGC-1α. The stimulation of the AMPK/SIRT1/PGC-1α axis results in the activation of transcription programs that increase the mitochondrial function and biogenesis.

5. Conclusion

This study shows that in human fibroblasts RSV activates transcriptional programs under the control of PGC-1α and p53, and up-regulates genes involved in FAO and mitochondrial biogenesis. Our data support the concept that RSV might be a relevant treatment in the correction of FAO deficiencies by increasing transcription of mutant mitochondrial β-oxidation enzymes with residual enzyme activities so that residual enzyme activity is raised above a critical lower threshold. Additionally RSV treatment may simultaneously have a cardioprotective effect in patients through increased RRAD expression. Moreover, the RSV mediated metabolic effects resulting from the modulation of the p53 pathway and from GNAS splicing could also be relevant contributors. We further demonstrate that RSV up-regulates SLC52a1 in normal fibroblasts and ASPA in normal and in ASPA-deficient patient fibroblast, in a dose- and time-dependent manner. While mutations in SLC52a1 have been related to transient forms of MADD, which can be restored with riboflavin supplementation, mutations in ASPA cause Canavan disease with no pharmacological treatment currently available. Because the most prevalent ASPA mutations result in low residual activity of the enzyme, we suggest that pharmacological treatment with RSV should be investigated, as even slight increases of enzyme activity could result in improved clinical phenotypes. Moreover, because RSV also affects the expression of POU3 factors that regulate transcriptional programs involved in myelination this could also be of benefit to patients with Canavan disease.

Competing interest statement: the authors have nothing to declare.

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Appendix A. Supplementary data
References


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