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Metabolite toxicity and DNA damage in Maple Syrup Urine Disease

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Abstract

Maple Syrup Urine Disease (MSUD) is a metabolic disease where the branched-chain amino acids (BCAAs) and their corresponding branched-chain α-keto acids (BCKAs) accumulates in the cells, because they have a defective branched-chain α-keto acid dehydrogenase (BCKD) complex, which is unable to degrade BCKAs. Norwegian patients are frequently defective in the E2 subunit of the BCKD. In several studies, the toxicity of accumulating BCAAs and BCKAs have been associated with oxidative stress and DNA damage, and therefore, it was investigated if fenofibrate and branched-chain fatty acids (BCFAs) could reduce the toxicity of BCAAs and BCKAs. Fenofibrate have been shown to reduce oxidative stress in previous studies, and because MSUD cells have been shown to lack BCFAs, treating MSUD cells with BCFAs might reduce the oxidative stress observed in the cells. The study aimed to determine if the toxicity of BCAAs and BCKAs are caused by DNA damage or by lack of downstream metabolites (e.g. BCFAs). Thus, the study investigated if fenofibrate and BCFAs could reduce oxidative stress and toxicity of BCAAs and BCKAs.

E2-deficient human haploid cells (HAP1) were used as model for MSUD, and repair-deficient mouse embryonal fibroblasts (MEF) were used to study the effect of DNA damage further. Both HAP1 and MEF cells were exposed to BCAAs and BCKAs and co-exposed to fenofibrate and BCFAs before they were studied in sensitivity assays and DNA damage analyses. Menadione-treatment was also used to study the effect of oxidative stress further, and to test if BCFA and fenofibrate reduced oxidative stress in cells.

Both WT and E2-deficient cells were more sensitive to BCKAs than BCAAs. The E2 subunit provided protection against menadione, but not against BCKAs. BCFA and fenofibrate made WT and E2-deficient cells equally sensitive to BCKAs, but BCFA and fenofibrate did not have any effect on menadione sensitivity. BCKAs had no significant effect on DNA damage in HAP1 cells. Repair-deficient cells were stimulated by BCKAs, and BCFAs restored normal sensitivity to BCKAs. The BCKAs induced nuclear DNA damage in repair-deficient MEFs.

The toxicity of BCKAs was not correlated to increased DNA damage levels. The E2 protein did protect cells against oxidative stress, but not against BCKAs. BCFAs and fenofibrate did not reduce oxidative stress in HAP1 cells, but they made WT and E2-deficient cells equally sensitive to BCKAs.

Sammendrag

Maple syrup urine disease (MSUD) er en metabolsk sykdom hvor forgrenede aminosyrer (BCAA) og deres korresponderende forgrenede α-ketosyrer (BCKA) akkumuleres i cellene, fordi branched-chain α-keto acid dehydrogenase (BCKD) komplekset ikke klarer å bryte ned BCKA. BCKD-subenheten, E2, er ofte mutert i norske MSUD pasienter. Flere studier har indikert en assosiasjon mellom toksisiteten til akkumulerende BCAA og BCKA med oksidativt stress og DNA skade, og derfor ble det undersøkt om fenofibrat og forgrenede fettsyrer (BCFA) kunne redusere toksisiteten av BCAA og BCKA. Tidligere studier har vist at fenofibrat reduserer oksidativt stress, og siden mangel av BCFA har blitt observert i MSUD-celler, vil BCFA-behandling av MSUD-celler kanskje redusere oksidativt stress i cellene. Formålet med studiet var å bestemme om toksisiteten til BCAA og BCKA forårsakes av DNA skade eller om den skyldes mangel av andre metabolitter (e.g. BCFA). Dermed ble det også undersøkt om fenofibrat og BCFA kunne redusere oksidativt stress og toksisiteten av BCAA og BCKA.

E2-defekte humane haploid-celler (HAP1) ble brukt som modell for MSUD, og reparasjonsdefekte mus-embryonale fibroblaster (MEF) ble brukt for å studere nærmere hvordan DNA-skade påvirker celler. Både HAP1- og MEF-celler ble eksponert for BCAA og BCKA, og samtidig behandlet med fenofibrat og BCFA, før de ble undersøkt med sensitivitetsassay og DNA-skade analyser. Menadione-behandling ble også brukt for å studere effekten av oksidativt stress nærmere, og for å se om BCFA og fenofibrate reduserte oksidativt stress i cellene.

Både WT og E2-defekte celler var mer sensitive til BCKA enn BCAA. E2-subenheten beskyttet mot menadione, men ikke mot BCKA. BCFA og fenofibrat gjorde WT og E2-defekte celler like sensitive til BCKA, men BCFA og fenofibrat hadde ingen effekt på menadione-sensitivitet. BCKA hadde ingen signifikant effekt på DNA-skade i HAP1-celler. Reparasjonsdefekte celler ble stimulert av BCKA, og BCFA gjenopprettet normal sensitivitet til BCKA. BCKA induserte nukleær DNA-skade i reparasjonsdefekte MEF.

BCKA-toksisiteten var ikke knyttet til økt DNA-skadenivå i cellene. E2-proteinet beskyttet cellene mot oksidativt stress, men ikke mot BCKA. BCFA og fenofibrat reduserte ikke oksidativt stress i HAP1-celler, men de gjorde WT og E2-defekte celler like sensitive til BCKA.

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1 Introduction

1.1 Maple Syrup Urine Disease

One of the most severe inherited disorders of amino acid metabolism is Maple Syrup Urine Disease (MSUD), which occurs in approximately 1 of 185 000 newborns. Affected individuals display symptoms like convulsions, apnea, hypoglycemia, coma, mental retardation and psychomotor delay (Sitta et al. 2014).

In cells, branched-chain amino acids (BCAAs) leucine, isoleucine and valine are converted to their respective branched-chain α-keto acids (BCKAs): α-ketoisocaproic acid, keto-β-methylvaleric acid, and α-ketoisovaleric acid. The α-keto acids are then decarboxylated to their CoA-derivatives by the enzyme branched-chain α-keto acid dehydrogenase (BCKD) (Chuang et al. 2006; Sperringer et al. 2017). The core BCKD complex consists of 3 subunits – E1, E2 and E3 (Chuang et al. 1984; Wynn et al. 1992).

In MSUD cells, however, a deficiency in the BCKD enzyme renders the enzyme unable to decarboxylate the α-keto acids, which leads to accumulation of BCAAs and BCKAs in the patients' plasma and tissues (Barschak et al. 2009; Chuang et al. 1982b; Dancis et al. 1959; Harris et al. 2004). The dihydrolipoamide branched chain transacylase E2 (*DBT*) gene, which encodes the E2 transacylase component, have been shown to contain mutations (Chuang et al. 2006). In 2010, one study found 4 new mutations in this gene in Norwegian MSUD patients (Brodtkorb et al. 2010). An outline for the catabolism of amino acids is shown in Figure 1.

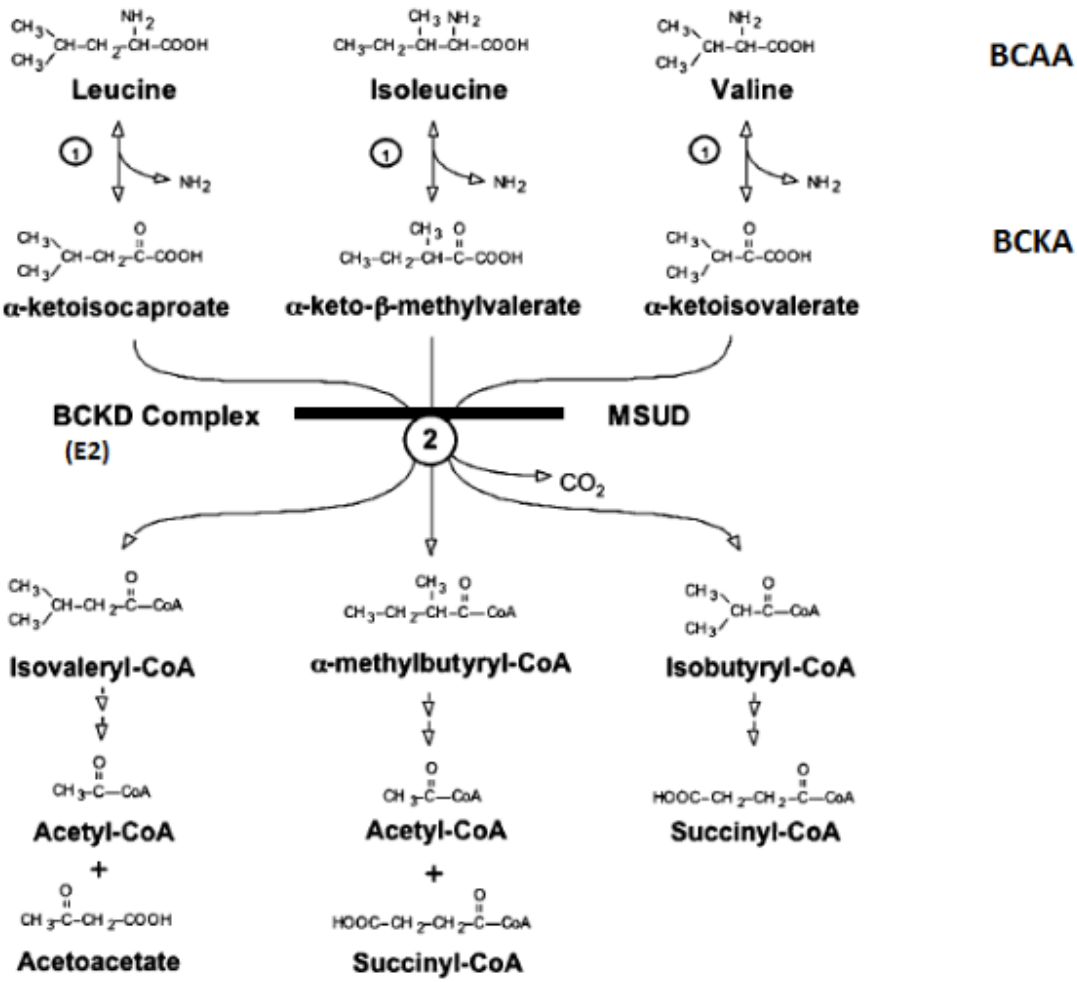


Figure 1. The catabolism of amino acids leucine, isoleucine and valine in cells. These amino acids are transaminated to their respective α-keto acids, before they are decarboxylated to CoA-derivatives by the BCKD enzyme. MSUD arises if the BCKD complex is impeded from degrading α-keto acids (Chuang et al. 2006). The figure is from Chuang et al. (2006).

Although the mechanisms behind the disease are not well known (Fontella et al. 2002), several features have been discovered in MSUD patients, like demyelation (Treacy et al. 1992), energy metabolism deficiency in the brain (Ribeiro et al. 2008; Sgaravatti et al. 2003), neuronal apoptosis (Jouvet et al. 2000), reduced levels of neutral amino acids (Araújo et al. 2001), increased lipid peroxidation and oxidative stress (Barschak et al. 2006; Barschak et al. 2008a; Barschak et al. 2008b; Bridi et al. 2003; Bridi et al. 2005a; Bridi et al. 2005b; Fontella et al. 2002; Funchal et al. 2006; Mescka et al. 2011), and DNA damage (Scaini et al. 2012; Strand et al. 2014).

1.2 Underlying mechanisms in MSUD

One of the features discovered in MSUD is oxidative stress. Oxidative stress is a cell condition where the levels of reactive oxygen species (ROS) exceeds the capacity of the antioxidant defense system (Halliwell 2007). When ROS production exceeds, they can create severe oxidative damage to lipids, proteins and DNA (Cui et al. 2012). Oxidative stress is considered important in many human disorders, like cancer, arteriosclerosis, diabetes mellitus, and neurodegenerative diseases (Li et al. 2014; Lin & Beal 2006; Sosa et al. 2013; Thanan et al. 2015; Tiwari et al. 2013).

DNA damage is one specific form of oxidative damage that has been discovered in MSUD. One study found that fibroblasts from MSUD patients had increased level of mitochondrial and nuclear DNA damage (Strand et al. 2014). Furthermore, acute BCAA exposure has been shown to increase DNA damage in the hippocampus, and that chronic exposure increased DNA damage in both hippocampus and striatum of rats. The same study also discovered that antioxidant treatment reduced the DNA damage content, implying that the cells in a rat model for MSUD experienced oxidative stress (Scaini et al. 2012).

Oxidative stress and DNA damage influence mitochondrial function (Van Houten et al. 2006). Mitochondrial DNA (mtDNA) accumulates more damage than nuclear DNA (nDNA), and mtDNA damage has been associated with mitochondrial dysfunction, such as ATP depletion and loss of membrane potential, which in turn lead to cell death. Therefore, mtDNA damage repair is crucial to the cells because it maintains mitochondrial function (Van Houten et al. 2006). Hence, although the nucleus encodes many of the proteins found in the mitochondria (Boengler et al. 2011), mtDNA is important for mitochondrial function. mtDNA is sensitive to oxidative stress and corresponding oxidative damage because mitochondria are the main site for ROS production in the cells (Cui et al. 2012; Paradies et al. 2015; Raha & Robinson 2000; Richter et al. 1995). The 2 main reasons for why mtDNA is particularly susceptible to ROS-induced damage are 1) that mtDNA is located close to the ROS, and 2) that mtDNA is not as well-protected as nDNA because it is not covered with histones (Yu & Bennett 2014; Yu & Bennett 2016; Ziech et al. 2011). As oxidative stress and DNA damage have been reported in MSUD, mitochondrial dysfunction might also be a crucial feature of this disease. One study indicated the presence of mitochondrial dysfunction in MSUD patients fibroblasts (Strand et al. 2014).

As of today, the treatment used for MSUD patients is a diet which reduces the BCAA intake and provides the patients with necessary amino acids, vitamins and minerals (Barschak et al. 2007; Barschak et al. 2008a; Lombeck et al. 1978). Although this treatment is beneficial in terms of preventing accumulation of BCAAs and BCKAs, the patients still show signs of mental retardation and developmental delay. Even if the restricted diet reduces the supply of proteins and nutrients, it can also potentially weaken the antioxidant system, which makes the cells more susceptible to oxidative stress (Barschak et al. 2007; Barschak et al. 2008b; Sitta et al. 2014). Because the treatment is unable to remove the symptoms completely, and as it can potentially increase oxidative stress in the cells, the prospect of new treatments is of interest.

Fenofibrate is a compound reported to reduce oxidative stress (Beltowski et al. 2002; Harano et al. 2006; Hou et al. 2010), but these findings are debated as several studies have reported opposite effects when applying fenofibrate (Nishimura et al. 2007; Zhao et al. 2013). Fenofibrate is a PPARα agonist (Berger et al. 2005), which increases mitochondrial β-oxidation (Berger et al. 2005; Staels et al. 1998). β-oxidation generates acetyl-CoA, which enters the citric acid cycle, and it generates NADH and $FADH_2$, which enters the electron transport chain (Houten & Wanders 2010). Therefore, fenofibrate might reduce the toxicity of BCAAs and BCKAs in MSUD via altered mitochondrial function that in turn influences oxidative stress and DNA damage.

Respiration is driven by catabolism of amino acids, fatty acids and carbohydrates (Fernie et al. 2004; Houten & Wanders 2010; Wu 2009). The latter two produce acetyl-CoA, which enters the citric acids cycle (TCA). In the TCA, NADH and $FADH_2$ are produced. Hence, fatty acid catabolism produces these electron carriers both in β-oxidation and indirectly via acetyl-CoA in the TCA (Fernie et al. 2004; Houten & Wanders 2010). NADH and $FADH_2$ transfer electrons to the electron transport chain (ETC), and as the electrons pass down the chain protein complexes, protons are moved from the mitochondrial matrix to the intermembrane space. This proton pumping creates an imbalance of positive charges over the membrane, establishing an electrochemical proton potential, negative on the inside. The electrochemical potential energy is used to generate ATP by coupling proton influx with mechanistical rotation of the ATP synthase (Complex V). This is called OXPHOS respiration (Barrientos 2002; Boyer 2002; Fernie et al. 2004; Jormakka et al. 2003). Oligomycin is an inhibitor of complex V (Cortez et al. 2012). The efficiency of respiration depends i.a. on the amount of proton leak present. Protons are able to venture across the inner mitochondrial

membrane through uncoupling proteins, and therefore, proton leaks reduce the proton motive force which in turn makes respiration less efficient (Jastroch et al. 2010).

Furthermore, MSUD have recently been suspected of being a deficiency disease, where the cells cannot produce branched-chain fatty acids (BCFAs) (Jia et al. 2016). In normal cells, BCKD catalyzes the degradation of BCKAs to BCFAs (Chuang et al. 2006; Harris et al. 2004), but when the BCKD enzyme is defective, BCFA cannot be synthesized from BCKA. Some types of BCFAs are 12-methyltetradecanoic acid (Yang et al. 2003), 15-methylpalmitic acid (Brooks et al. 2009), and 18-methyleicosanoic acid (Jones & Rivett 1997). The requirement for BCKD in biosynthesis of BCFA is demonstrated by the lack of BCFA in MSUD (Jones & Rivett 1997). Therefore, lack of BCFAs might partly explain the toxicity of BCAAs and BCKAs in MSUD.

1.3 Tools to identify the toxic compounds in MSUD

Oxidative stress and DNA damage have been observed in many studies, and therefore, it would be possible to assume that these features represent the underlying mechanisms of the toxicity of BCAAs and BCKAs. Of the two metabolite types, BCKAs have been shown to be more toxic than BCAAs (Jouvet et al. 2000). Many studies have reported increased lipid peroxidation and reduced antioxidant defenses, which indicates the presence of oxidative stress in the cells (Barschak et al. 2006; Barschak et al. 2008a; Barschak et al. 2008b; Bridi et al. 2003; Bridi et al. 2005a; Fontella et al. 2002). Studies on DNA damage and MSUD have observed increased DNA damage in MSUD models (Scaini et al. 2012; Strand et al. 2014). One of these studies found that increased mtDNA and nDNA damage in MSUD fibroblasts, where BCKAs were suspected of being the DNA damaging substance (Strand et al. 2014). Therefore, it must be determined if DNA damage is the main cause of BCKA toxicity in MSUD.

Several cell types have been used to study MSUD. Strand et al. (2014) used fibroblasts from patients, while other have used brain and neuronal cells from rats (Bridi et al. 2003; Bridi et al. 2005a; Jouvet et al. 2000; Ribeiro et al. 2008; Sgaravatti et al. 2003). In this study, human haploid (HAP1) cells were used as a model for the disease because MSUD is an autosomal recessive disorder (Chuang et al. 1982a). As HAP1 cells are haploid, they have only one copy of each gene (Essletzbichler et al. 2014). A HAP1 cell line (E2) with crispr-knockout of the

DBT gene was used, because it might be able to replicate the MSUD phenotype as the gene encoding the important E2 transacylase in the BCKD has been inactivated.

Additionally, repair-deficient mouse embryonal fibroblasts (MEFs) were used because they have been used extensively in studies on oxidative stress and DNA damage (Arai et al. 2002; de Souza-Pinto et al. 2001; Klungland et al. 1999; Liu et al. 2011; Osterod et al. 2001). This knockout cell line lacks the 7,8-dihydro-8-oxoguanine DNA glycosylase (OGG1) (Klungland et al. 1999). OGG1 is an important base excision repair (BER) enzyme as it removes one of the most frequent lesion in mammalian genome, 7,8-dihydro-8-oxoguanine (8-oxoG) lesions (Barzilai & Yamamoto 2004; de Souza-Pinto et al. 2001; Klungland et al. 1999; Powell et al. 2005), and mouse cells lacking the OGG1 enzyme have been shown to contain increased amounts of DNA damage compared to WTs (Arai et al. 2002; de Souza-Pinto et al. 2001; Liu et al. 2011). BER repairs oxidative DNA lesions both in the mitochondria and in the nucleus (Weissman et al. 2007).

As BER is important for mtDNA repair, it is crucial for cell survival because it maintains the mitochondrial function (Van Houten et al. 2006; Weissman et al. 2007). It would therefore be interesting to study the BCAAs and BCKAs in cells that have compromised DNA repair. In this study, mitochondrial DNA damage and mitochondrial function are the crucial features of the repair-deficient MEF model, and this might also be the case for MSUD HAP1 cells.

To determine if the toxicity of BCAAs and BCKAs in MSUD is caused by DNA damage, the toxicity of BCAAs and BCKAs was first studied with cell sensitivity assays. Then, DNA damage was quantified to confirm if the BCAA/BCKA sensitivity observed in the cells correlated with the DNA damage content. As it is uncertain whether BCAAs and BCKAs induce mitochondrial dysfunction in HAP1 cells, it would be useful to compare the toxicity of the BCAAs and BCKAs with a chemical that is known to induce mitochondrial dysfunction and DNA damage in cells through inducing oxidative stress. This way, it could be determined if the E2 and OGG1 proteins protect cells against oxidative stress. Menadione was selected as the stress-inducing chemical because it is known to increase the ROS content of cells (Loor et al. 2010; Richter et al. 1995), which in turn increases oxidative stress that triggers mitochondrial dysfunction (Halilovic et al. 2016; Marchionatti et al. 2008).

Additionally, the effect of fenofibrate and BCFAs on BCAA/BCKA toxicity and oxidative stress was investigated. Fenofibrate was chosen because it has been reported to reduce oxidative stress in some studies (Beltowski et al. 2002; Harano et al. 2006; Hou et al. 2010),

and because it should affect mitochondrial function through affecting respiration. BCFA was also chosen because lack of BCFA has been discovered in MSUD (Jia et al. 2016), and because the BCKD have been shown to be required in the BCFA synthesis (Jones & Rivett 1997). Fenofibrate and BCFA might affect reactions and functions which are important for cell survival, and because oxidative stress has frequently been associated with MSUD, fenofibrate and BCFA might alter cell sensitivity towards BCAAs and BCKAs by affecting the cells' resistance to oxidative stress.

The effect of fenofibrate on mitochondrial function in cells experiencing menadione-induced stress was further examined. Because fenofibrate is a PPARα agonist (Berger et al. 2005), it can also influence transcription as PPARαs are transcription factors (Hill et al. 1999; Kersten et al. 1999), and therefore, it should affect mitochondrial function through modifying respiration and gene expression. These mitochondrial parameters are affected by DNA damage, which implies that mitochondrial function is also dependent on the DNA damage content in cells (Van Houten et al. 2006). Therefore, DNA damage might be considered a mitochondrial parameter. The effect of fenofibrate on oxidative stress was therefore investigated in WT MEFs, an immortalized cell model that is much studied in the laboratory (Klungland et al. 1999), by analyzing respiration, gene expression and DNA damage.

Together, this study hypothesizes that accumulated BCAA or BCKA leads to increased DNA damage, which in turn triggers MSUD via mitochondrial dysfunction, and that fenofibrate and BCFA increase survival of E2-deficient cells, where fenofibrate improves mitochondrial function and additional BCFA reduces the DNA damage content/oxidative stress.

1.4 Aims of the study

1. The role of DNA damage produced by BCAAs and BCKAs

To investigate the relationship between DNA damage and the toxicity of BCAA and BCKA.

2. The role of fenofibrate in cell sensitivity of E2-deficient cells

To investigate alterations in cell sensitivity towards BCAAs and BCKAs caused by fenofibrate.

3. The role of BCFA in cell sensitivity of E2-deficient cells

To investigate alterations in cell sensitivity towards BCAAs and BCKAs caused by BCFA.

2 Materials

Mouse embryonic fibroblasts (MEFs) from WT and 8-oxoguanine DNA glycosylase knockout (*Ogg1*$^{-/-}$) mice, strain c57/bl.6 were from our own collection (Klungland et al. 1999). The cell genotypes were verified with gene expression analysis (Figure A in the Appendix).

Human near-haploid cells (HAP1s, WT) (from Horizon) were from the strain male chronic myelogenous leukemia (CML) cell line KBM-7. A E2 mutant generated by CRISPR technique (from Horizon), which lacks the branched-chain keto acid transacylase (E2). The cell genotypes were verified with Western Blot analysis (data not shown).

Table 1. Oligonucleotide primers used in this study for DNA damage and gene expression analysis

Species	Target	Forward primer (5'-3')	Reverse primer (5'-3')
Human	*mt-RNR1* (Taq)	aaactgctcgccagaacact	catgggctacaccttgacct
	NDUFA9 (Taq)	gcaagggtccctatgagagaa	caagaacgaggggaaaagtg
Mouse	*mt-Rnr1* (Taq)	actcaaaggacttggcggta	agcccatttcttcccatttc
	Ndufa9 (Taq)	ctcaagtccattgaggtgct	gaccgaatcctcggatattt
	Gapdh	tcgtcccgtagacaaaatggt	cgcccaatacggccaaa
	Eno1	gctcgcgtctgtccttaag	agatctctctggcgtggatc
	Pgk1	ccatgcctgacaagtactcc	aggcattctcgacttctggg
	Tpi1	agaagtgcctgggagaactc	tggcaaagtcgatgtaagcg
	mt-Rnr1 (12S)	actcaaaggacttggcggta	agcccatttcttcccatttc
	Tfam	gcaatgtggagcgtgctaaaa	tgctggaaaaacacttcggaata
	mt-Nd6 (Nd6)	aacaaccaaccaaaaaggctta	gctgggtgatctttgtttgc
	Ogg1	gtgactacggctggcatcc	aggcttggttggcgaagg
	Neil1	gctgaccctgagccagaagat	ccccaactggaccacttcct

3 Methods:

3.1 Cell cultivation and treatments

The cells were grown in cultivation medium which consisted of Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, cat. number 6429) supplemented with 10% Gibco fetal bovine serum (FBS) (Thermo Fisher Scientific, cat. number 10270106) and 1% Penicillin/Streptomycin supplement (Lonza, cat. number 17-602E). The cells were grown in either T75 (cat. number 156499) or T175 (cat. number 159910) flasks from Nunc/Thermo Fisher Scientific. The confluency of the HAP1 cells was kept below 75% to avoid diploidism, according to the manufacturer's recommendation. The cell count of HAP1s and MEFs was determined with Countess Automated Cell Counter (Invitrogen), using Countess™ Cell Counting Chamber Slides (Invitrogen, cat. number C10283). Equal volumes of cell suspension and Trypan Blue (Invitrogen, cat. number T10282) was mixed in an Eppendorf tube, and 11 μL was transferred to the slide.

The cells were routinely checked for mycoplasma contamination. Mycoplasma detection was performed with a MycoAlert™ Mycoplasma Detection Kit (Lonza, cat. number LT07418) according to the manufacturer's Assay protocol. The detection is based on the ability of the specific mycoplasma enzymes to convert ADP to ATP after adding a specific substrate, and the ATP is coupled to luciferin/luciferase detection system. If the ATP concentration is increased, it will generate light that can be quantified in a luminometer (*Lonza - Mycoplasma Detection Kit Protocol* 2011).

Respiration analysis was performed with MEFs grown to a confluency of 40-90%, and gene expression analysis was performed with MEFs grown to a confluency of 40-60%. Cell confluency prior to DNA damage analysis on MEFs varied between 20 and 70%. The cells were grown in tissue culture dishes (cat. number 353003) from Corning/VWR. HAP1 cells were also grown in these dishes prior to DNA damage analysis.

Trypsin (Sigma-Aldrich, cat. number T4049) was used to harvest the cells for respiration, DNA and RNA analyses. PBS (phosphate-buffered saline, Rikshospitalet) was used to remove the serum-containing medium that otherwise would inactivate trypsin, and was also used to remove serum-containing medium from harvested samples to ensure that the medium did not interfere with the process of isolating DNA and RNA. The samples were centrifuged at 3000

rpm for 5 minutes before they were washed twice with PBS. After each washing, the cells were centrifuged at 4000 rpm for 1 minute, and the supernatant was removed. The cell pellets were stored at -80°C.

Menadione treatment was performed by exposing the cells to menadione (Sigma-Aldrich, cat. number M5750) for 1 hour in cultivation medium at 37°C. 100 mM menadione stocks were prepared in nuclease-free water (Ambion/Applied Biosystems, cat number AM9937), sterile filtrated, and used either (preferably) fresh or within 2 weeks.

When applicable, the cells were subjected to treatment with fenofibrate (Sigma-Aldrich, cat. number F6020) or alternatively BCFAs 1 day prior to treatment with menadione and simultaneously to treatment with BCAAs and BCKAs. 100 mM stocks of fenofibrate and BCFAs (12-methyltetradecanoic acid and 15-methylpalmitic acid) (Sigma-Aldrich, cat. numbers M3664, M6531) were prepared in DMSO (Sigma-Aldrich, cat. number D8418) and sterile filtrated. Fenofibrate stocks was used within 2 weeks and BCFA stocks within 4 weeks.

The BCAA stock consisted of 10 mM of each of the amino acids leucine, isoleucine and valine (Sigma-Aldrich, cat. numbers L8912, I7403, V0513) dissolved in cultivation medium. The BCKA stock contained 50 mM of each of the branched-chain α-keto acids: α-ketoisocaproic acid, keto-β-methylvaleric acid, and α-ketoisovaleric acid (Sigma-Aldrich, cat. numbers K0629, 198994, 198978), dissolved in cultivation medium. The stocks were sterile filtrated and used within 4 weeks.

3.2 Nucleic acid isolation and quantification.

Total DNA was isolated from cultured cells with a Qiagen Blood and Tissue Kit according the manufacturer's protocol with certain modifications: When the samples were incubated for 10 minutes at 56°C, they were also continually mixed at 450 rpm. Nuclease-free water was used instead of elution buffer (Buffer AE) to elute the cells, and the volume used varied depending on the size of the cell pellet. Moreover, the solutions were pipetted up and down instead of vortexing.

Total RNA was isolated from cultured cells with a Qiagen RNeasy mini kit according to the manufacturer's protocol with certain modifications. In step 1 of “RNA cleanup”, the samples were not adjusted to 100 µL with RNase-free water, and in addition to Buffer RLT, 6 µL β-

ME (2-mercaptoethanol) (Sigma-Aldrich, cat. number M3148) was added to the samples. Furthermore, 300 µL of 70% ethanol (Antibac/Kemetyl, cat. number 600068) was used instead of 250 µL of 96-100% ethanol. The optional "On-column DNase digestion" was performed before continuing on step 4 of "RNA cleanup".

Nucleic acid concentrations were estimated using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific), NanoDrop One (Thermo Fisher Scientific) and Epoch Microplate Spectrophotometer (BioTek). DNA and RNA absorb ultraviolet light (UV) at 260 nm (Fleige & Pfaffl 2006). A purity measure is indicated by the ratio (absorbance at 260nm/absorbance at 280nm), where pure RNA has a ratio of 2.0 and pure DNA has a ratio of ≥1.8 (Desjardins & Conklin 2010). The difference in purity ratio is caused by the ability of the DNA double helix to prevent UV absorbance to a greater degree than the single-stranded RNA (Watson et al. 2014, p. 89-90).

3.3 Real-time quantitative PCR (RT-qPCR)

Quantitative real time PCR was performed with StepOnePlus2.2.2 (Applied Biosystems/Thermo Fisher Scientific) using the Power SYBR Green PCR Master mix (Applied Biosystems/Thermo Fisher Scientific, cat. number 4367659). RT-qPCR was used for assessing DNA damage and gene expression levels, as well as testing the primer quality and was estimated with comparative Ct (cycle threshold) values (ΔΔCt).

3.3.1 DNA damage analysis

The method was established previously by Wang et al. (2016). The template DNA concentration was adjusted to 20 ng/µL instead of 10 ng/µL for nDNA damage analysis, and the qPCR program used 95°C instead of 94°C. The qPCR master mix set up is described in Table 2. The total volume of each qPCR reaction was 10 µL, which consisted of 3 µL DNA and 7 µL of the qPCR master mix.

Table 2. The qPCR master mix (MM) for DNA damage quantification

	MM_{NT}	MM_{Taq}	Final concentration of 10 µL
Nuclease-free water	1 µL	0.975 µL	-
Power Sybr Green master mix (2x)	5 µL	5 µL	1x
TaqαI (20.000 U/mL)	-	0.025 µL	0.5 U
Forward primers (10 µM)	0.5 µL	0.5 µL	0.5 µM
Reverse primers (10 µM)	0.5 µL	0.5 µL	0.5 µM

RT-qPCR amplifies a specific part of DNA defined by the primers for the gene chosen for analysis. The restriction enzyme, TaqαI (New England Biolabs, cat. number R0149S/L), cuts DNA in the TaqI restriction site. DNA damage in the restriction site, impedes TaqI digestion, leaving a DNA strand that can be amplified and quantitated in RT-qPCR. The nondamaged DNA will be cleaved by TaqI and can consequently not serve as template in RT-qPCR. The DNA damage content is determined by the difference in efficiency to amplify a qPCR signal from sample template DNA versus reference template DNA, as, ΔCt = Ct (sample) – Ct (reference). The damage frequency = 1/(2^ΔCt), therefore indicates the frequency of damaged TaqI sites to non-damaged (Wang et al. 2016).

DNA damage was assessed in the mitochondrial ribosomal gene *mt-Rnr1* and in the nuclear gene *Ndufa9* in mice and in the corresponding orthologues in humans. The primers sequences are listed in Table 1.

3.3.2 Gene expression analysis

Gene expression analysis starts with preparing cDNA from RNA. Synthesis of cDNA involves using primers that bind random locations along the RNA strands. Then, the reverse transcriptase uses dNTPs to extend the primers to produce cDNA from these regions (Kubista et al. 2006).

cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Thermo Fisher Scientific, cat. number 4368814). First, based on the RNA concentrations provided by Nanodrop, 40 ng/µL RNA solutions were made with nuclease-free water to a total volume of 10 µL in PCR strips. Then, 10 µL of the cDNA Reverse Transcription master mix was added, and they were subjected to reverse trancription-PCR (RT-PCR) with Mastercycler®pro (Eppendorf) using the program designed by the manufacturer. The cDNA Reverse Transcription master mix was made according to the manufacturer's protocol for Kit without RNase Inhibitor (*Applied Biosystems - Protocol for cDNA Reverse Transcription* 2010).

After cDNA had been prepared, 3 µL cDNA was mixed with 7 µL of the qPCR master mix described in Table 3, so the total volume of each qPCR reaction was 10 µL. The RT-qPCR program was almost the same as the program used for DNA damage quantification. The only difference was that the 65°C step was removed.

Table 3. The RT-qPCR master mix (MM) for gene expression analysis

	MM	Final concentration of 10 µL
Nuclease-free water	1 µL	-
Power SYBR Green master mix (2x)	5 µL	1x
Forward primers (10 µM)	0.5 µL	0.5 µM
Reverse primers (10 µM)	0.5 µL	0.5 µM

The gene expression data was subjected to relative quantification analysis, where the data were normalized for differences in RNA input by comparing the expression of genes to an internal control (*Gapdh*) (ΔCt) before the replicates were averaged. The normalized gene expression for each sample was subsequently compared relative to the normalized average control sample (NT) (ΔΔCt). Lastly, the fold change (2^-ΔΔCt) was determined and averaged. The fold change represents how much the expression of genes in treated samples differs from the non-treated sample (Livak & Schmittgen 2001).

The primer sequences used for expression analysis are listed in Table 1. The housekeeping gene, *Gapdh*, was used as an endogenous (internal) control. The mitochondrial genes *mt-Rnr1* (*12s*) and *mt-Nd6* (*Nd6*), and the nuclear genes *Eno1*, *Pgk1*, *Tpi1*, *Tfam*, *Ogg1* and *Neil1* were investigated.

3.3.3 Primer testing

Primer testing is important to ensure that the primers are suitable for use in analysis. Primers are considered efficient if their maximal amplification efficiency is close to the theoretical amplification efficiency. The theoretical amplification efficiency (100%) implies that 3.32 PCR cycles are needed to make a 10-fold increase in the DNA copy number (*Relative Quantitation of Gene Expression (RT-qPCR)* 2008). Primer efficiency is determined by preparing serial dilutions of cDNA. For each dilution, cDNA is diluted with the same factor, and therefore, more PCR cycles are needed to reach the cDNA concentration that results in a fluorescent signal. Hence, the Ct value increases. An optimal primer will follow a linear relationship between the log cDNA concentration and the Ct values, but they are inversely proportional. If a primer deviates from the linear relationship between cDNA content and Ct value, then it is considered unsuitable for analysis (Heid et al. 1996; *Real-time qPCR: primer efficiency* 2016).

Primer testing was performed by preparing 5-fold serial dilutions of cDNA dissolved in nuclease-free water. Nuclease-free water was also used as a negative control. The protocol for preparing cDNA and the RT-qPCR procedure is described in “Gene expression analysis”.

3.4 Mitochondrial respiration analysis

Mitochondrial respiration was measured by Oroboros Oxygraph-2k (Oroboros) equipped with Clark electrodes, where different compound can be added to the cells to evaluate the cellular conditions through respiration (Gnaiger 2008; Gnaiger 2011).

2 mL cell suspension (0.5-2 million cells) was added to the chambers, which were sealed, and enabling the cells to consume dissociated oxygen in the air-tight chambers (Doerrier & Gnaiger 2016). Then, the cells were treated with 2.5 μM oligomycin (Sigma-Aldrich, cat.

number O4876), a complex V inhibitor, to measure the level of respiration that was independent of complex V (Cortez et al. 2012; Pesta & Gnaiger 2012). Following complex V inhibition, the cells were uncoupled with stepwise addition of 0.5 μM FCCP (Sigma-Aldrich, cat. number C2920) to dissipate the proton motive force (PMF), which reveals the maximum respiration capacity (Gnaiger et al. 1998; Pesta & Gnaiger 2012).

3.5 Cell sensitivity analysis

According to the Roche manual, the MTT assay is based on the ability of the cells' metabolic enzymes to reduce the tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), to a formazan derivative (Berridge & Tan 1993; Edmondson et al. 1988), which can be quantitated spectrophotometrically at 550-600 nm after it has been solubilized by a solution agent (*Roche - Cell Proliferation Kit I (MTT) Protocol* 2016). Formazan and non-converted MTT absorb light similarly, at wavelengths higher than 650 nm, which then can be used as a reference wavelength (*Roche - Cell Proliferation Kit I (MTT) Protocol* 2016).

Cell sensitivity was determined with a Cell Profileration Kit I (MTT) (Roche/Sigma-Aldrich, cat. number 11465007001) and the absorbance was measured with Epoch Microplate Spectrophotometer (BioTek) in the program Gen5 2.01. The cell viability was quantified by the absorbance of reduced MTT (*Roche - Cell Proliferation Kit I (MTT) Protocol* 2016) and presented as relative to non-treated value. MEF cells (10 000-50 000 pr. well) and HAP1 cells (10 000 pr. well) were plated in a 96 well plate (Corning/Sigma-Aldrich, cat. number CLS3595). They were either left untreated or pre-treated with fenofibrate or BCFA for 1 day prior to menadione exposure. The BCAA/BCKA-treated cells were exposed to fenofibrate or BCFA simultaneously as BCAA and BCKA was added to the cells. The 96 well plates were incubated for 2 (MEF) and 4 (HAP1) days. After their respective incubation periods, the solutions were removed, and the fluorescent dye, MTT, was prepared with cultivation medium (10%). The cells were treated with MTT (10%) for 4 hours before a solution agent was added to disrupt the cells and solubilize the formazan salts. The solution agent had a 1:1 relationship with MTT (*Roche - Cell Proliferation Kit I (MTT) Protocol* 2016). Next day, the reduced MTT was quantitated at 570 nm, and the non-converted MTT (and formazan) at 750 nm. Long incubation periods were used to make sure the cells grew to a density that provided a signal in the spectrophotometer. This way, the incubation periods corrected for the cells that

died of treatment immediately versus those that survived, and the periods helped to correct for cells that stop proliferating (senescence).

3.6 Statistical analysis

All experiments were performed at least three independent times. The results are presented as mean±SE. Student's t-test was performed to evaluate significance, where p values less than 0.05 and 0.01 are denoted by * and **, respectively.

4 Results:

4.1 Investigating the toxicity of accumulating amino acids versus keto acids

As the E2-deficient HAP1 cells lack the BCKD enzyme, they might be susceptible to accumulated BCAAs and BCKAs. The toxicity of BCAAs and BCKAs was determined by treating cells with 0, 2, 4 and 6 mM BCAA or BCKA for 4 days and measuring the survival with an MTT assay.

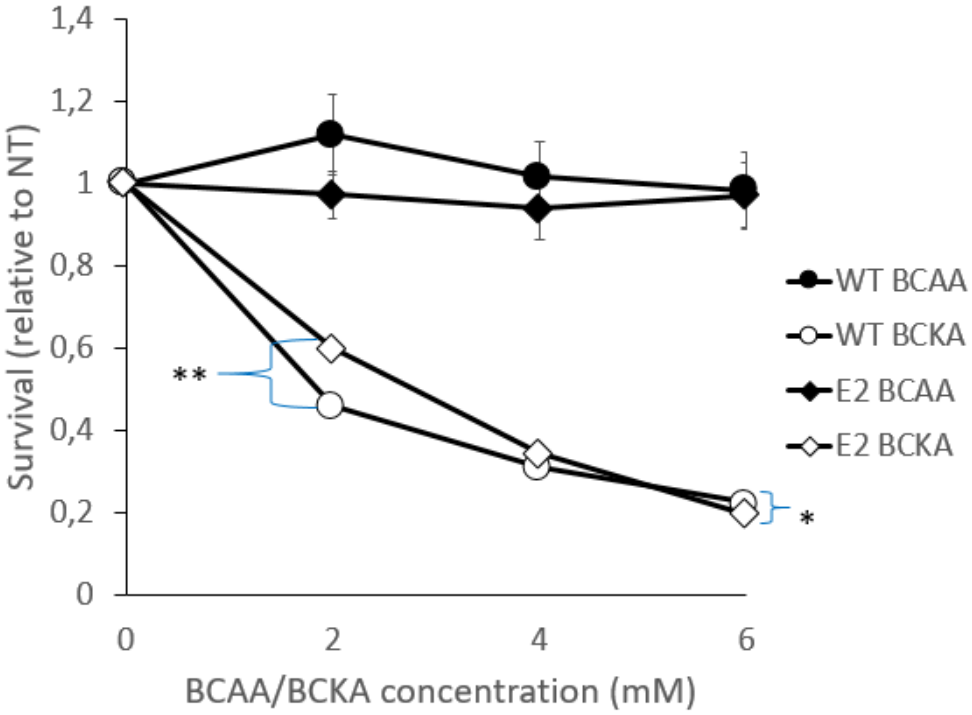
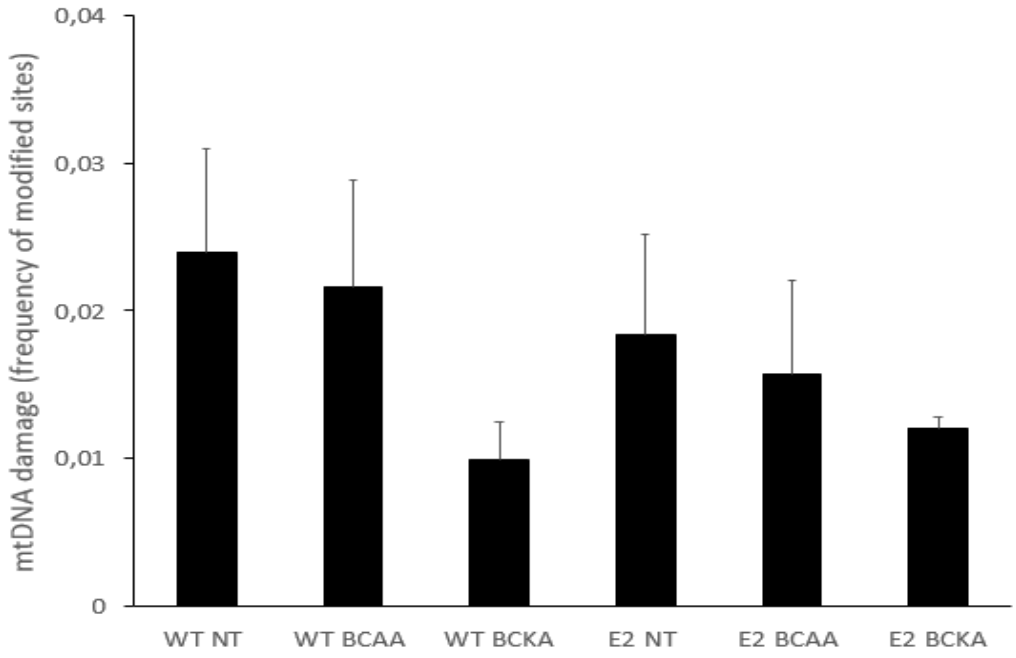


Figure 2. HAP1 sensitivity to BCAA and BCKA. HAP1 sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were incubated with BCAA or BCKA in cultivation medium for 4 days (37°C) (N=4) (All error bars are present, but some are too low to be visible in the figure). The stars (*,) and the brackets indicate significant difference between WT and E2-deficient cells. * p<0.05, **p<0.01.**

Figure 2 demonstrates that BCKAs were more toxic than BCAAs ($p<0.01$), but that E2-deficient cells were less sensitive to 2 mM BCKA than WT. E2-deficient cells were shown to be significantly more sensitive than WT to 6 mM BCKA, but this might be caused by saturation of the signals (see section 5.1).

To determine if DNA damage could be the cause of BCKA toxicity, DNA damage analysis was performed on DNA isolated from cells treated with BCAAs and BCKAs. WT and E2-deficient cells were exposed to either 10 mM BCAA or 10 mM BCKA in cultivation medium for 1 day before the cells were harvested and analyzed (Figure 3).

A



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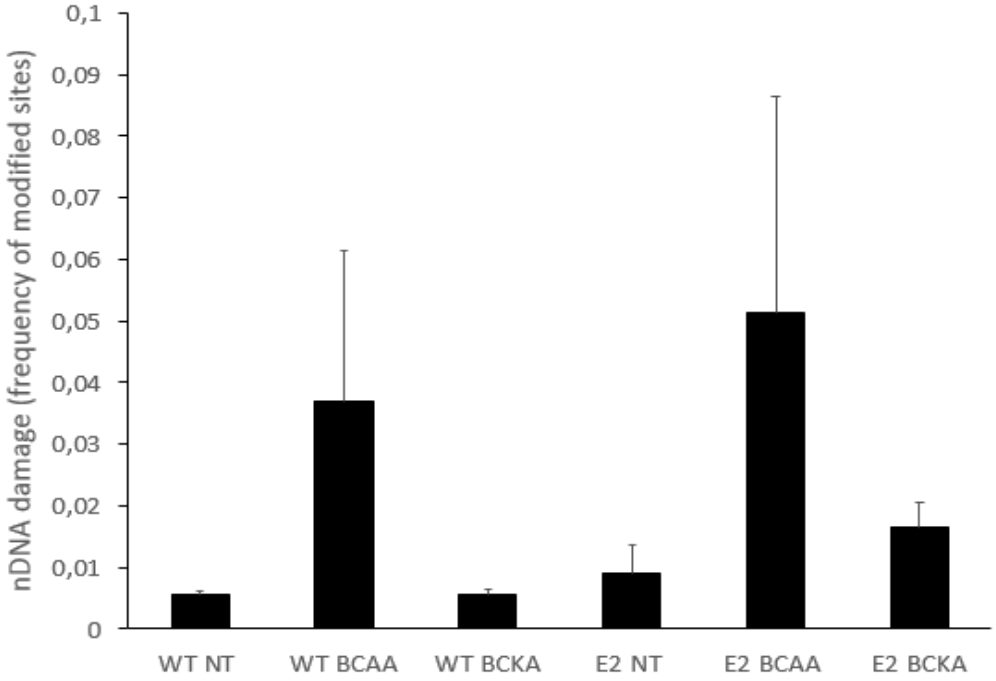


Figure 3. mtDNA and nDNA damage in cells treated with BCAA and BCKA. HAP1 DNA damage was assessed by restriction enzyme inhibition as described in Methods and provided as the frequency which TaqI cuts DNA. The HAP1 cells were treated with BCAA or BCKA in cultivation medium at 37°C for 1 day before the medium was removed and the cells were harvested for DNA isolation and damage analysis. A = mtDNA, B = nDNA (N=3).

In Figure 3, the DNA damage contents followed 2 different trends. First, BCKA reduced mtDNA damage (Figure 3A), and secondly, BCAA increased nDNA damage (Figure 3B). The mtDNA and nDNA damage levels were quite similar between WT and E2-deficient cells.

As such, BCKA did not significantly affect the level of DNA damage in the E2-deficient cells. Hence, no correlation was found between the DNA damage content and BCKA toxicity. However, BCKA might induce other forms of oxidative damage which might culminate in mitochondrial dysfunction and cell death. Menadione is a chemical known to increase the ROS content of cells (Loor et al. 2010; Richter et al. 1995), which in turn increases oxidative stress that triggers mitochondrial dysfunction (Halilovic et al. 2016; Marchionatti et al. 2008). Menadione-toxicity was therefore investigated in WT and E2-deficient HAP1 cells to study if the E2 protein protected cells against oxidative stress (Figure 4). Figure 4 indicates that the E2 protein did protect HAP1 cells against oxidative stress as the WT was significantly less sensitive to 100 and 125 μM menadione than E2-deficient cells.

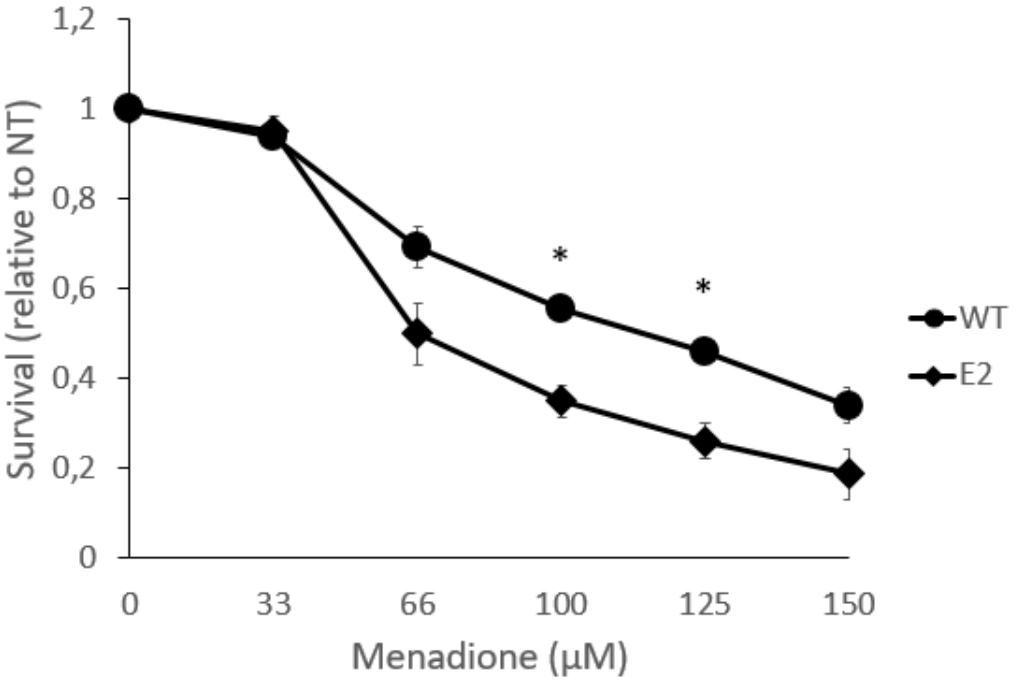


Figure 4. HAP1 sensitivity to menadione. HAP1 sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were exposed to menadione in cultivation medium at 37°C for 1 hour before the medium was replaced, and the cells were grown further for 4 days and analyzed (N=3) (All error bars are present, but some are too low to be visible in the figure). The stars (*) indicates significant difference between WT and E2-deficient cells. * p<0.05.

4.2 Investigating fenofibrate as potential treatment for oxidative stress

4.2.1 Investigating respiration in menadione-treated WT MEFs

Since menadione exerts a specific effect in E2-deficient cells, it was important to characterize the impact of menadione on mitochondrial function, exemplified by respiration capacity. WT cells were exposed to 40 µM menadione in cultivation medium at 37°C for 1 hour before they recovered for 2 and 4 hours in menadione-free medium. If the induced oxidative damage alters respiration, recovery is a measure of how well cells repair oxidative damage to restore normal respiration.

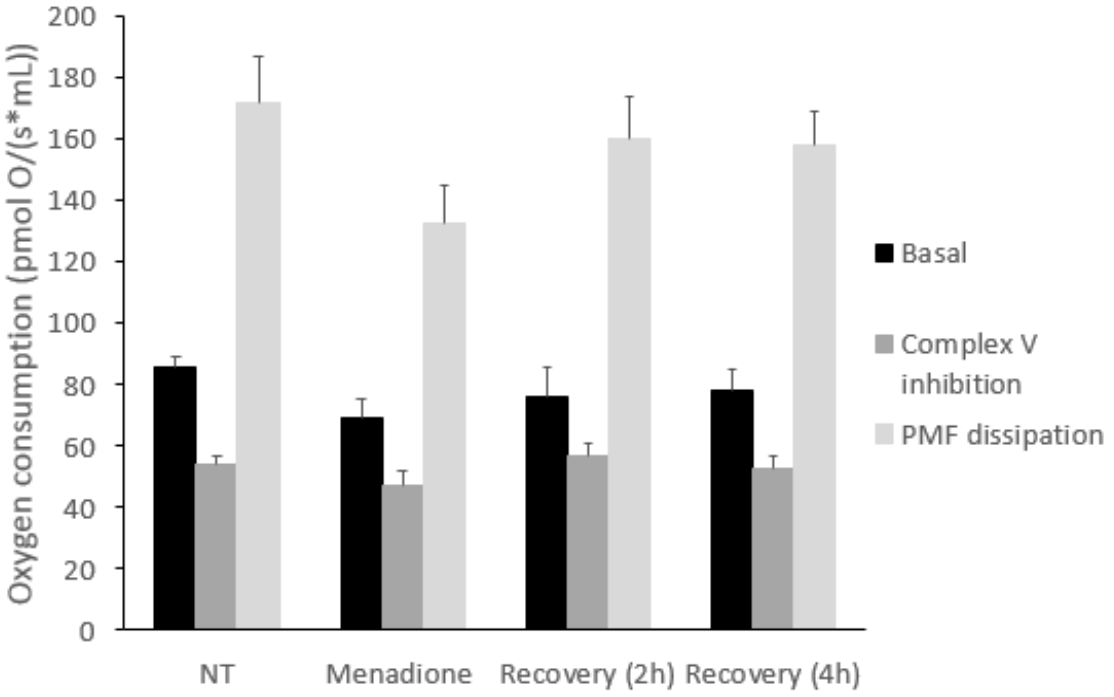


Figure 5. Respiration in WT MEF cells treated with menadione. Respiration analysis was performed as described in Methods. Oxygen consumption in WT MEF cells treated with menadione for 1 hour in cultivation medium at 37°C before the medium was replaced. The cells then recovered for 2 and 4 hours before they were harvested for analysis. ~1-2 million cells were used in the analysis. Complex V inhibition refers to oligomycin treatment, and PMF (proton motive force) dissipation refers to FCCP treatment (N=4).

Menadione did not significantly alter cellular respiration, neither in short term nor up to 4 hours of post-treatment under the conditions studied here (Figure 5).

4.2.2 Investigating respiration, gene expression and DNA damage in fenofibrate- and menadione-treated WT MEFs

As the E2-deficient cells are sensitive to menadione-induced stress, it was investigated if fenofibrate could improve cellular sensitivity in E2-deficient cells to oxidative stress by altering mitochondrial function. The WT MEFs were subjected to respiration, gene expression

and DNA damage analysis, where menadione was used to induce oxidative stress. The WT MEFs underwent the same treatment prior to each analysis, where they were pre-treated with 25 µM fenofibrate 1 day in cultivation medium at 37°C prior to menadione exposure (40 µM, 1h). The cells were also treated with only menadione (40 µM, 1h) or fenofibrate (25 µM, 1 day) to study their effects alone compared to the co-treated sample.

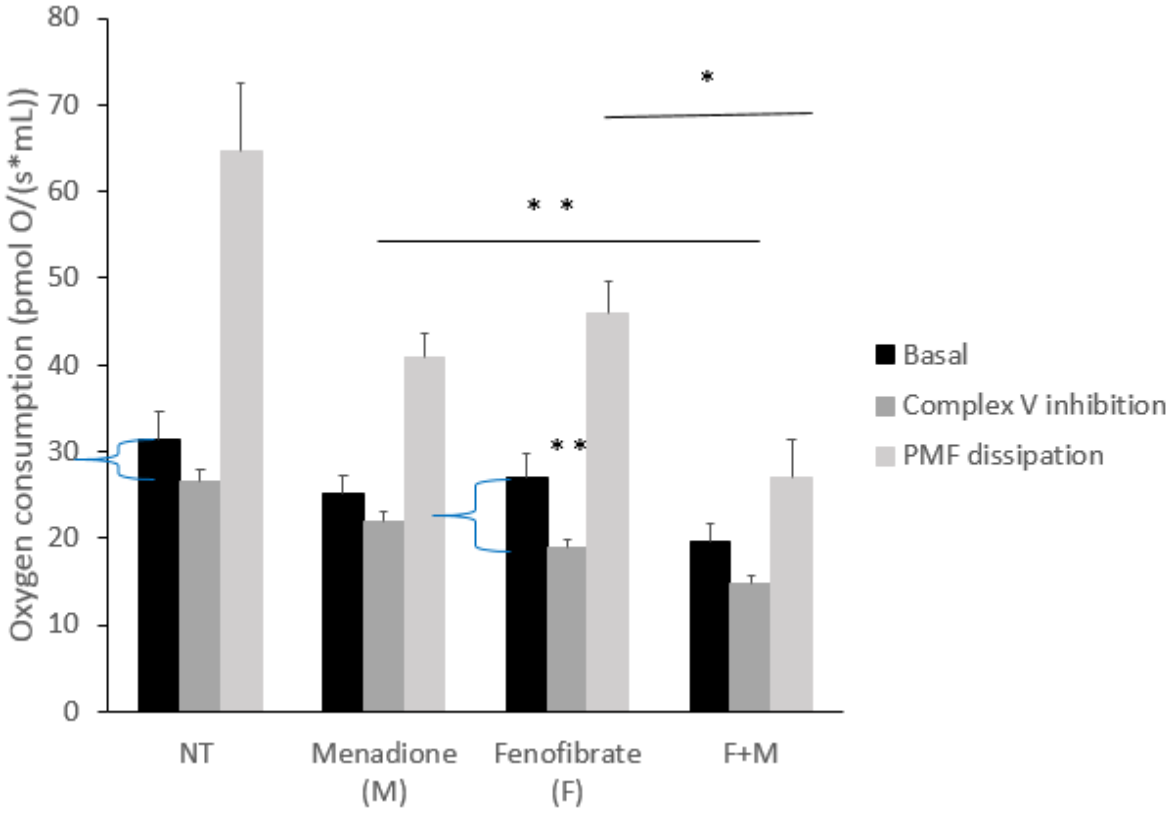


Figure 6. Respiration in WT MEF cells treated with fenofibrate and menadione. Respiration analysis was performed as described in Methods. Oxygen consumption in WT MEF cells treated with menadione (1h), fenofibrate (1 day), or pre-treated with fenofibrate 1 day prior to menadione exposure (1h), all in cultivation medium at 37°C. To finish treatment, the medium was removed and the cells were harvested for analysis. In this analysis, ~450 000-800 000 cells were used. The brackets indicate the OXPHOS-dependent respiration in the control and in the fenofibrate sample. The menadione and fenofibrate sample were compared to the control (NT) and significance are indicated with stars (**). The co-treated sample were compared to the fenofibrate and menadione sample, and in both cases, when the relationship is significant, it is indicated by stars (*,) over a line between the two columns in question. Complex V inhibition refers to**

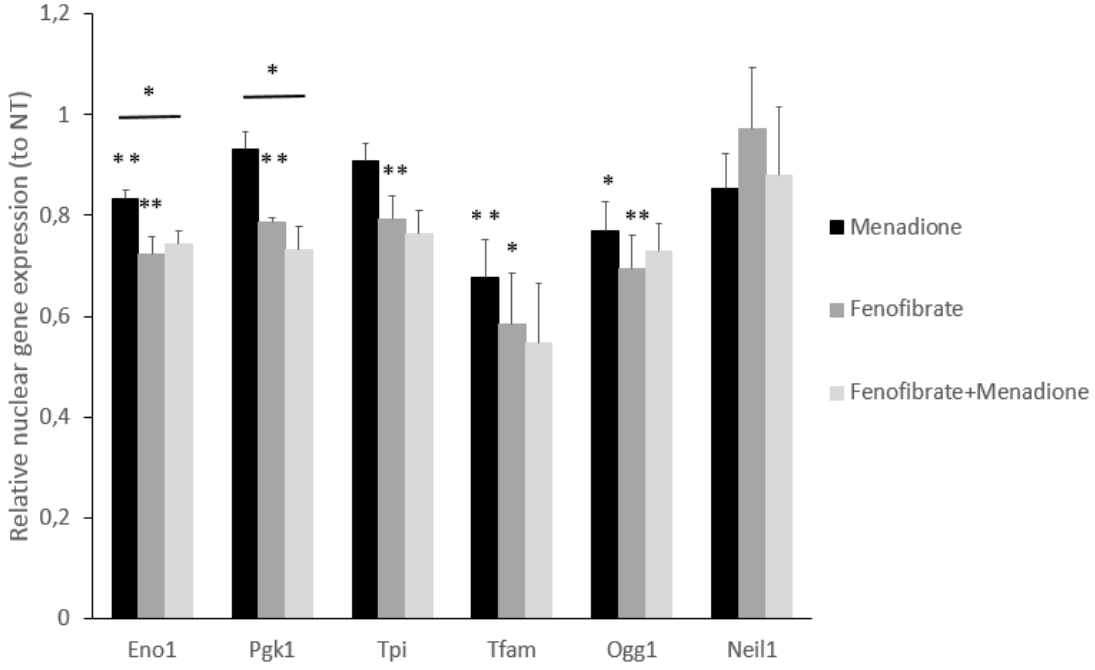
oligomycin treatment, and PMF (proton motive force) dissipation refers to FCCP treatment (N=4). * p<0.05, **p<0.01.

Figure 6 shows that respiration was unaffected by menadione. On the other hand, fenofibrate reduced oxygen consumption in WT MEFs when complex V of the electron transport chain was inhibited by oligomycin. In other words, fenofibrate increased the respiration (OXPHOS) efficiency (as indicated by the blue brackets in the figure) because it reduced OXPHOS-independent respiration. Hence, the leak of protons across the inner mitochondrial membrane was reduced (Jastroch et al. 2010). The trend also indicates that fenofibrate reduces the respiration capacity in cells in the present conditions.

Co-treated cells displayed increased respiration efficiency compared to menadione-treated cells, which implies that fenofibrate was the main agent behind this difference in efficiency. On the other hand, co-treated cells had reduced respiration capacity compared to fenofibrate-treated cells, which implies that the maximum oxygen consumption was reduced when menadione was present.

Next, the effect of fenofibrate and menadione on nuclear and mitochondrial gene expression in WT MEFs was determined. Glycolytic genes (*Eno1, Pgk1, Tpi1*) and *mt-Nd6* were chosen as they are essential in respiration (Grzybowska-Szatkowska & Ślaska 2014; Hauf et al. 2000; Ji et al. 2016; Pirola et al. 2013). Genes (*Ogg1, Neil1*) encoding DNA repair enzymes were also chosen as they protect DNA against mutations and thereby stimulates mitochondrial function (David et al. 2007). Additionally, genes important for mitochondrial biogenesis (*Tfam, mt-Rnr1*) were used. TFAM is a transcription factor that regulates transcription of mitochondrial DNA, and *mt-Rnr1* encodes mitochondrial 12s ribosomal RNA, which is important for ribosome production (Byun et al. 2013; Istiaq Alam et al. 2003).

A



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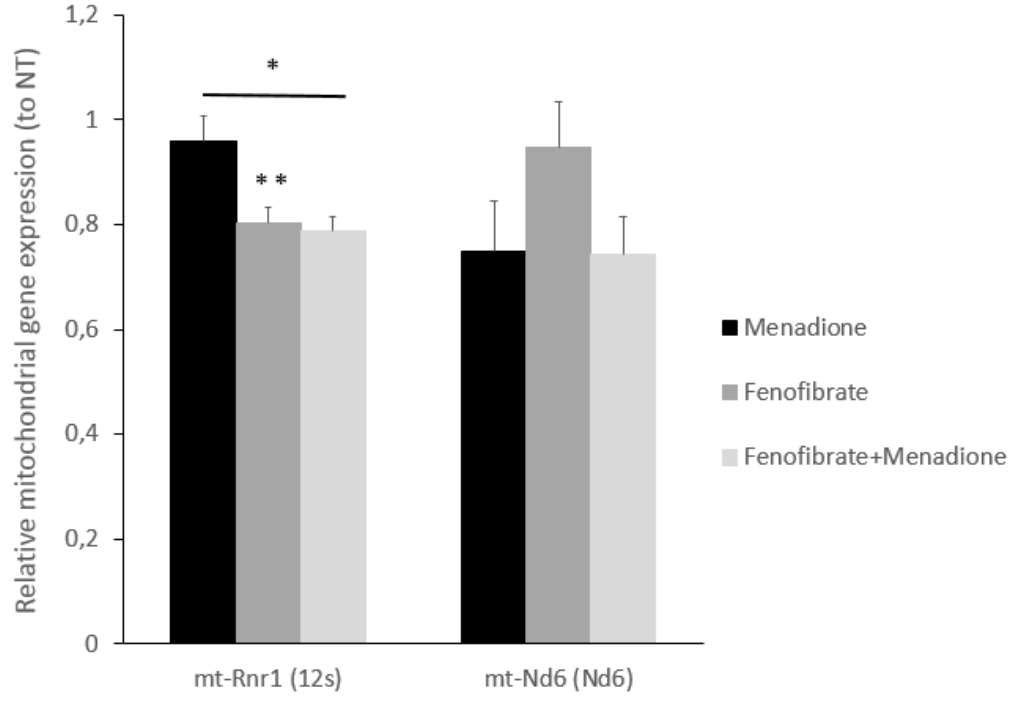


Figure 7. Relative expression of mitochondrial and nuclear genes in WT MEFs treated with fenofibrate and menadione. Gene expression was assessed as described in Methods. The WT cells were treated with menadione (1h), fenofibrate (1 day), or pre-treated with fenofibrate 1 day prior to menadione exposure (1h), all in cultivation medium at 37°C.

To finish the treatment, the medium was removed and the cells were harvested for further analysis. The housekeeping gene, *Gapdh*, was used as internal control. All the untreated samples were set to 1. The menadione and fenofibrate sample were compared to the control (NT) and significance are indicated with stars (*,). The co-treated sample were compared to the fenofibrate and menadione sample, and in both cases, when the relationship is significant, it is indicated by stars (*) over a line between the two columns in question. A: nuclear genes, B: mitochondrial genes (N=4). * p<0.05, **p<0.01.**

In WT MEFs, fenofibrate reduced expression of glycolytic genes (*Eno1*, *Pgk1*, *Tpi1*), *Ogg1*, *Tfam* (Figure 7A), and *mt-Rnr1* (Figure 7B). Menadione reduced expression of *Eno1*, *Tfam* and *Ogg1*, and co-treated cells showed reduced expression of *Eno1*, *Pgk1* and *mt-Rnr1* when compared to the menadione-treated cells. On the other hand, no difference was observed between co-treatment and fenofibrate treatment. The expression of *mt-Nd6* and *Neil1* was not significantly altered by any of the treatments, although the trend indicates that fenofibrate increased expression of these genes.

Finally, DNA damage analysis was performed to investigate if fenofibrate reduced oxidative DNA damage in menadione-exposed cells. The level of mitochondrial DNA damage in the *mt-Rnr1* gene in WT MEFs was assessed by the TaqI inhibition method devised by Wang et al. (2016).

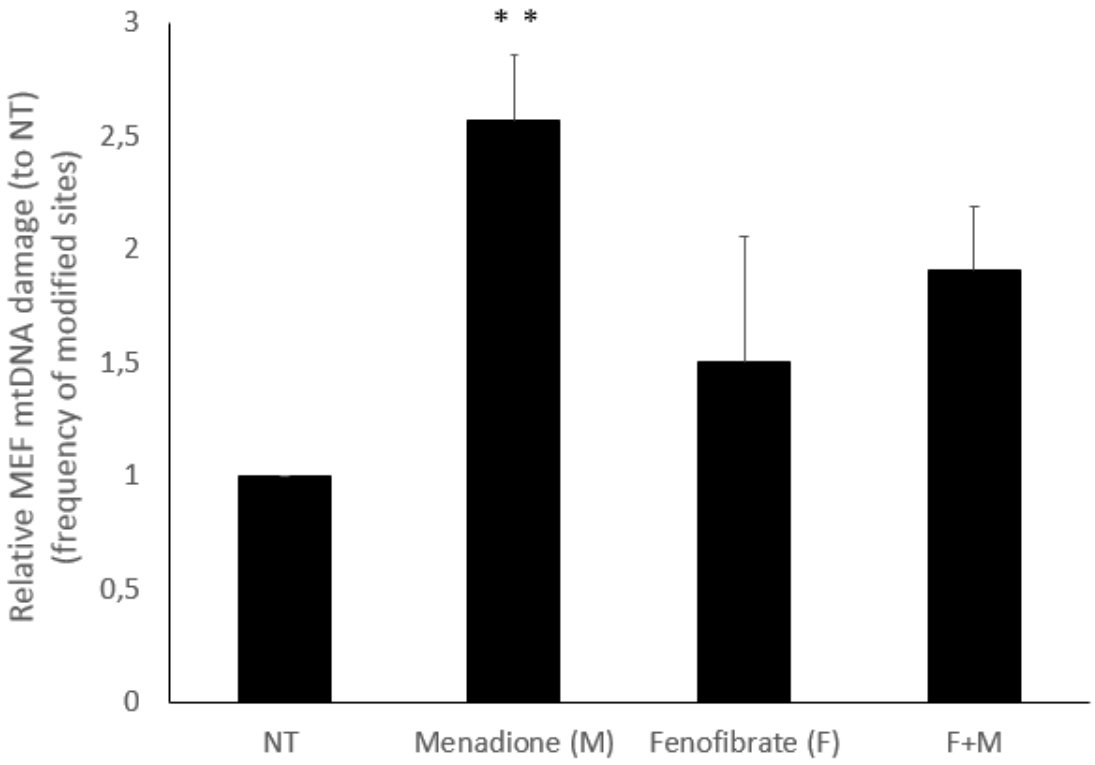


Figure 8. Relative mtDNA damage in fenofibrate- and menadione-treated WT MEFs. DNA damage was assessed by restriction enzyme inhibition as described in Methods. mtDNA damage was determined as treated samples relative to a control (NT). The cells were treated with menadione (1h), fenofibrate (1 day), or pre-treated with fenofibrate 1 day prior to menadione exposure (1h), all in cultivation medium at 37°C. To finish the treatment, the medium was removed, and the cells were harvested for DNA damage analysis. The menadione and fenofibrate sample were compared to the control (NT) and significance is indicated with stars (**). The co-treated sample were compared to the fenofibrate and menadione sample (N=4). **p<0.01.

As shown in Figure 8, menadione induced DNA damage in WT MEFs, while fenofibrate did not significantly reduce DNA damage induced by menadione. The trend suggests that fenofibrate increased DNA damage to a certain extent, and that the co-treated sample had a DNA damage content between the menadione and fenofibrate sample.

Taken together, fenofibrate increased the respiration efficiency, and together, fenofibrate and menadione reduced the respiration capacity. Expression of genes involved in glycolysis, DNA damage repair, mitochondrial transcription and ribosome production was reduced after

fenofibrate treatment. Menadione induced DNA damage, while fenofibrate did not significantly alter the DNA damage content in non-treated or menadione-treated cells.

4.3 Repair-deficient MEFs as a tool for evaluating the cellular impact of DNA damage

In the previous section, WT MEFs was only used as a tool to establish the effect of fenofibrate on cells and on oxidative stress. In this section, the relationship between BCAA/BCKA toxicity and DNA damage will be further analyzed in repair-deficient MEFs. Later, it will be investigated if fenofibrate reduces the toxicity of BCAAs and BCKAs in both E2-deficient and repair-deficient cells.

Repair-deficient MEFs accumulate more DNA damage than WT MEFs as their repair system lack the OGG1 DNA glycosylase (Arai et al. 2002; de Souza-Pinto et al. 2001; Liu et al. 2011). Because their DNA repair system is compromised, repair-deficient MEFs might be more sensitive to BCKAs than WTs if the BCKAs induce DNA damage in MEFs. The cellular impact of DNA damage induced by BCAAs and BCKAs could therefore be evaluated by comparing BCAA/BCKA toxicity and DNA damage levels in repair-deficient MEFs.

MEF survival was determined by treating the cells with 0, 5, and 10 mM BCAA or BCKA for 2 days before treating them with MTT (Figure 9).

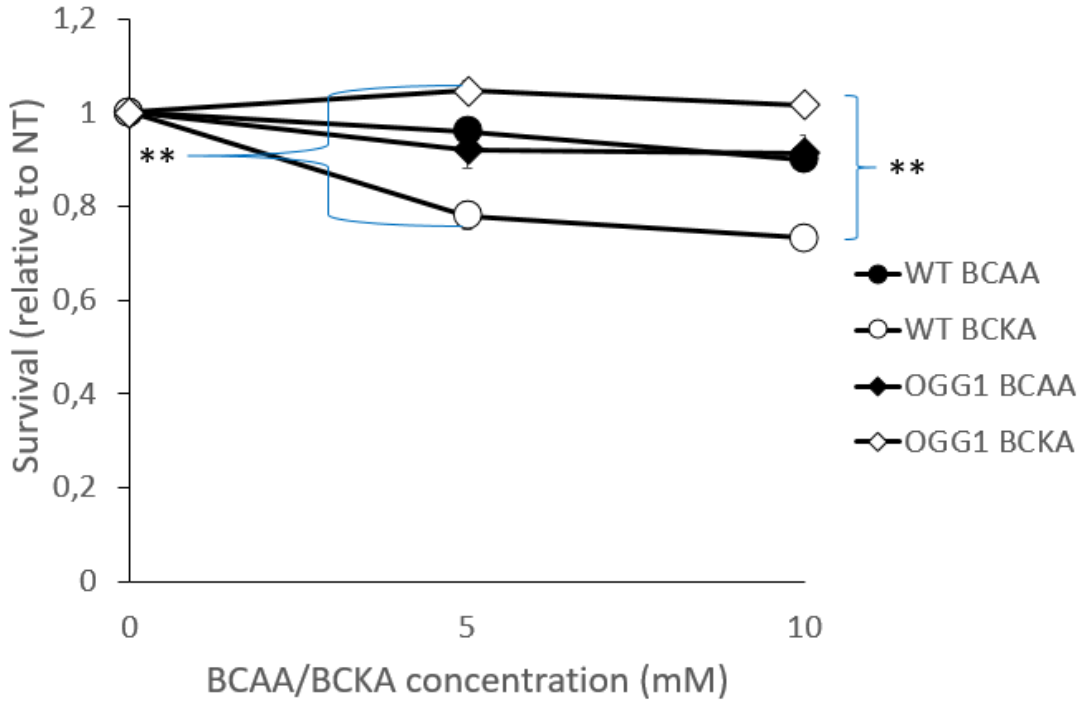
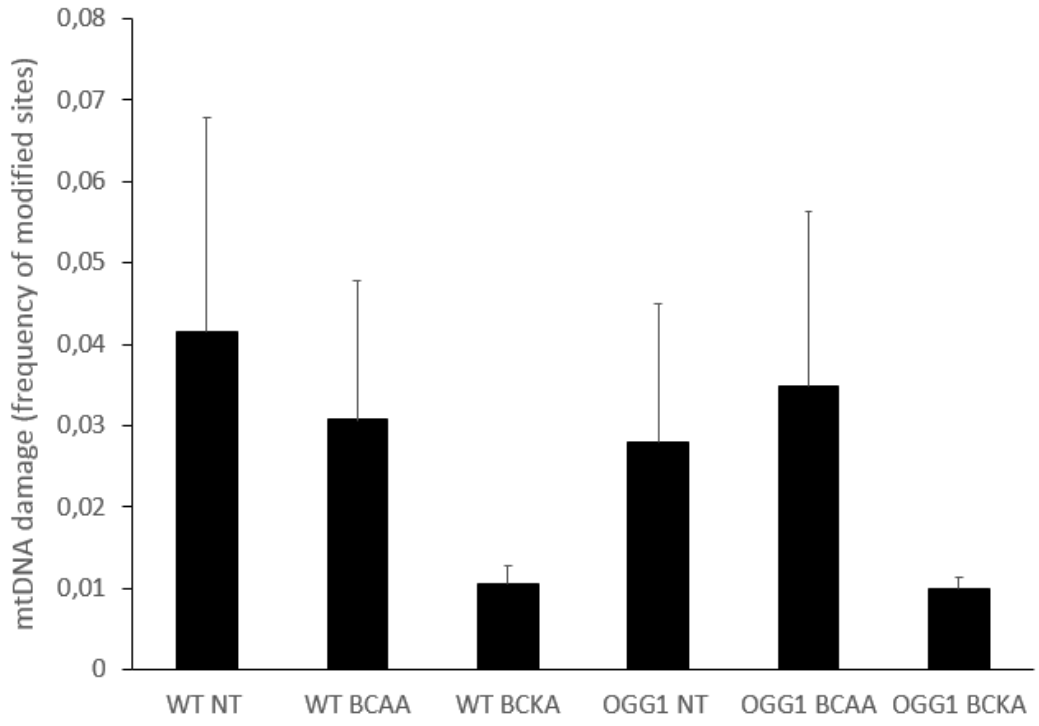


Figure 9. MEF sensitivity to BCAA and BCKA. MEF sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were incubated with BCAA or BCKA for 2 days in cultivation medium at 37°C (N=4) (All error bars are present, but some are too low to be visible in the figure). The stars (**) and the brackets indicate significant difference between WT and repair-deficient cells. **p<0.01.

WT MEFs were more sensitive to BCKA than repair-deficient cells (Figure 9), while BCKA appeared to be well tolerated by OGG1-deficient MEFs. WT MEFs were also found to be more sensitive to BCKA than BCAA ($p<0.01$). OGG1-deficient cells, on the other hand, were more sensitive to BCAA than BCKA when the concentration was 5 mM ($p<0.05$).

Then, the level of DNA damage was determined to investigate if the DNA damage content correlated with survival in BCAA/BCKA-treated repair-deficient cells. The cells were exposed to either 10 mM BCAA or 10 mM BCKA for 1 day before the cells were harvested for damage analysis on mitochondrial and nuclear DNA (Figure 10).

A



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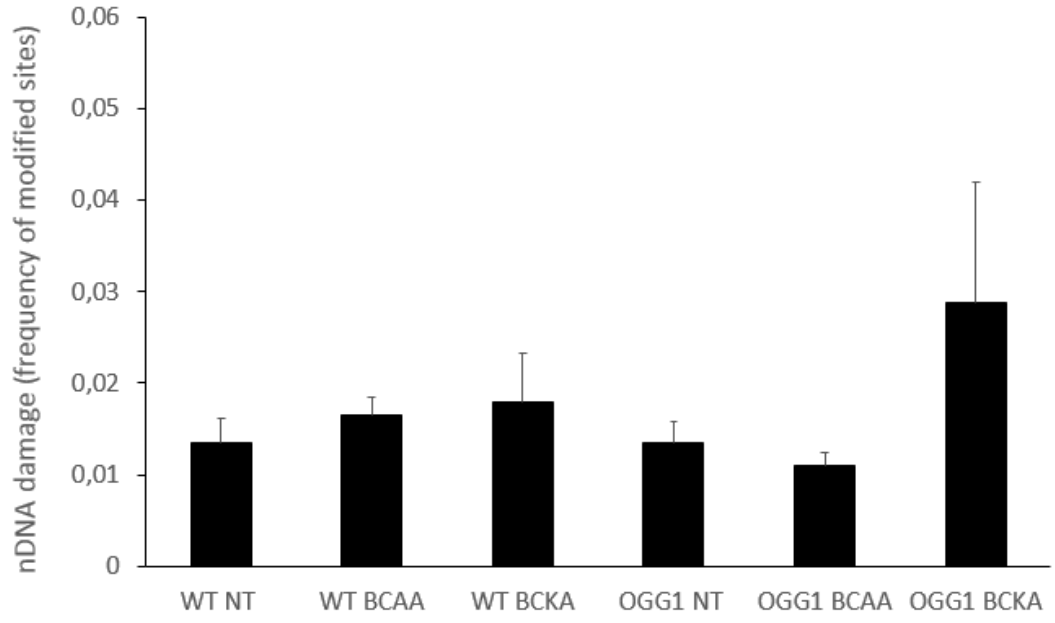


Figure 10. mtDNA and nDNA damage in MEFs treated with BCAA and BCKA. MEF DNA damage was assessed by restriction enzyme inhibition as described in Methods and provided as the frequency which TaqI cuts DNA. The MEF cells were treated with BCAA or BCKA for 1 day in cultivation medium at 37°C before the medium was

removed and the cells were harvested for DNA isolation and damage analysis. A = mtDNA, B = nDNA (N=3).

The trends in Figure 10 indicates that BCKA reduced mtDNA damage in WT and repair-deficient cells, and increased nDNA damage in repair-deficient cells. BCAA did not alter the DNA damage content in either of the genotypes.

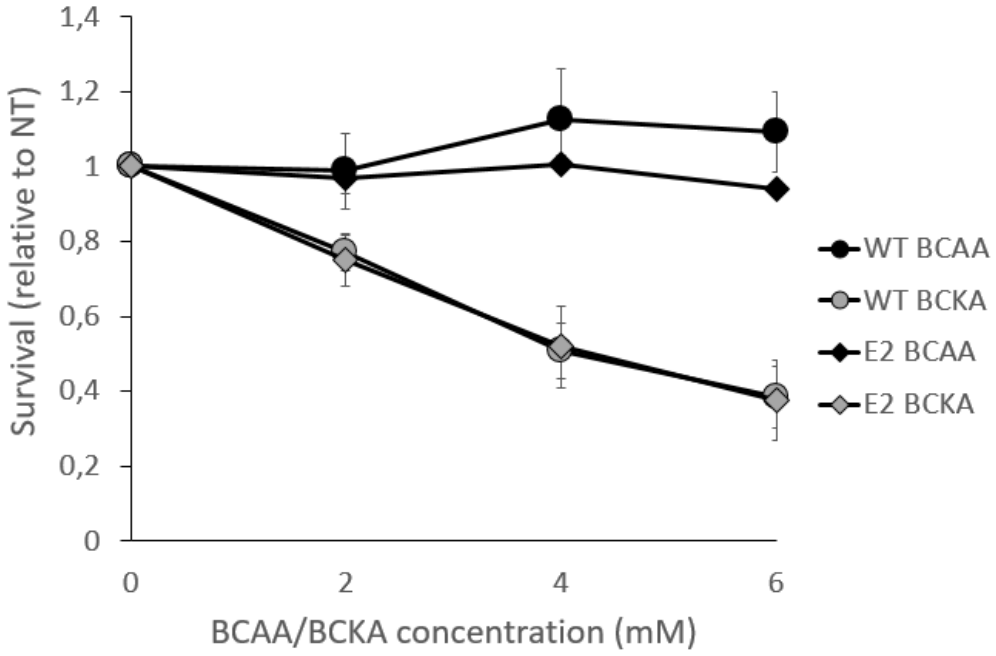
No correlation between BCKA toxicity and DNA damage was observed in MEFs, as the mtDNA damage content was low in WT MEFs and as the nDNA damage content was high in repair-deficient MEFs. However, BCKA might induce other forms of oxidative damage by inducing oxidative stress. Menadione-toxicity was therefore investigated in WT and repair-deficient MEFs to study if the OGG1 enzyme protected cells against oxidative stress (Figure 15). Figure 15 indicates that the OGG1 enzyme was not important for protecting MEFs, as WT and repair-deficient cells responded equally well to menadione.

4.4 The effect of fenofibrate and BCFA on BCAA/BCKA toxicity

4.4.1 Investigating if BCFA or fenofibrate alters HAP1 sensitivity to BCAAs and BCKAs

This study also aimed to evaluate the effect of BCFA and fenofibrate on BCAA/BCKA toxicity in HAP1s. BCAA/BCKA-toxicity in HAP1 cells was determined in cells treated for 4 days with 0, 2, 4 and 6 mM BCAA or BCKA under continuous co-administration of either 25 μM BCFA or 25 μM fenofibrate. To discover if fenofibrate or BCFA influenced cell growth, samples treated only with these compounds were compared to the control. Neither BCFA nor fenofibrate stimulated cell growth separately.

A



B

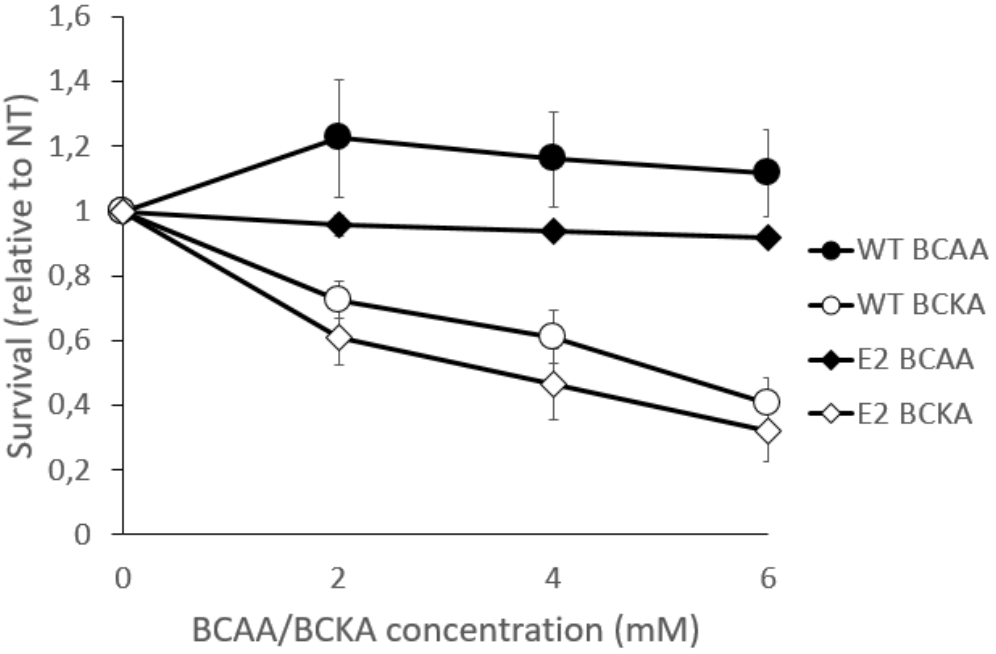


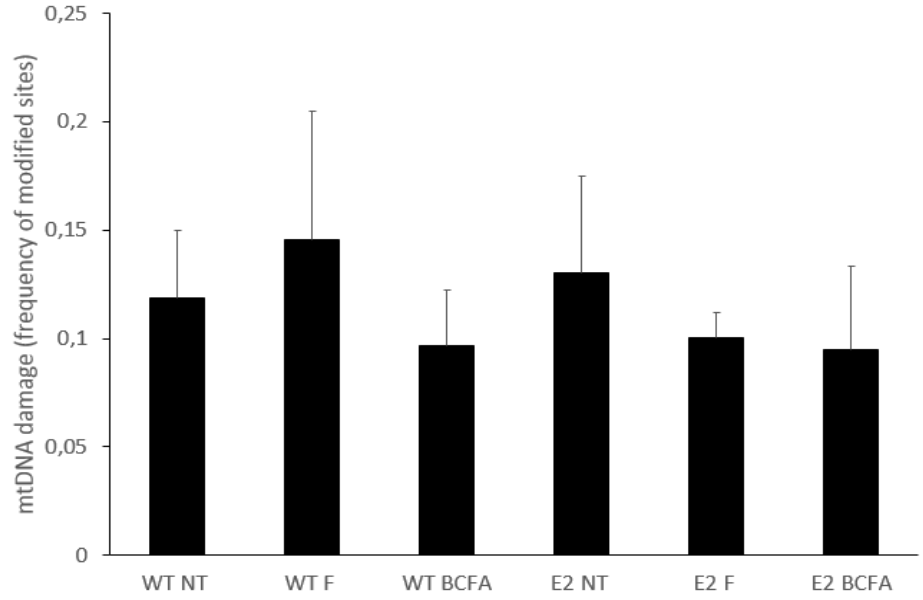
Figure 11. HAP1 sensitivity to BCAA and BCKA. HAP1 sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were incubated with BCAA or BCKA, and simultaneously exposed to either (A) BCFA or (B) fenofibrate, respectively. The cells were grown in cultivation medium at 37°C for 4 days (N=3) (All error bars are present, but some are too low to be visible in the figure).

Figure 11 shows that WT and E2-deficient cells were equally sensitive to both BCAA+BCFA and BCKA+BCFA. The combination of BCKA+BCFA was more toxic than treatment with BCAA+BCFA (4, 6 mM, $p<0.05$). BCKA+fenofibrate was more toxic than BCAA+fenofibrate to WT when the concentration was increased (6 mM, $p<0.05$), and to E2-deficient cells at all concentrations (2, 4 mM, $p<0.05$; 6 mM, $p<0.01$).

Compared to Figure 2, BCFA and fenofibrate eliminated the significant difference between WT and E2-deficient cells treated with 2 mM BCKA.

Then, DNA damage analysis was performed on cells treated with either fenofibrate (25 μM, 1 day) or BCFA (25 μM, 1 day) to study whether these chemicals reduced the level of DNA damage in HAP1 cells that were not exposed to oxidative stress-inducing substances (Figure 12).

A



B

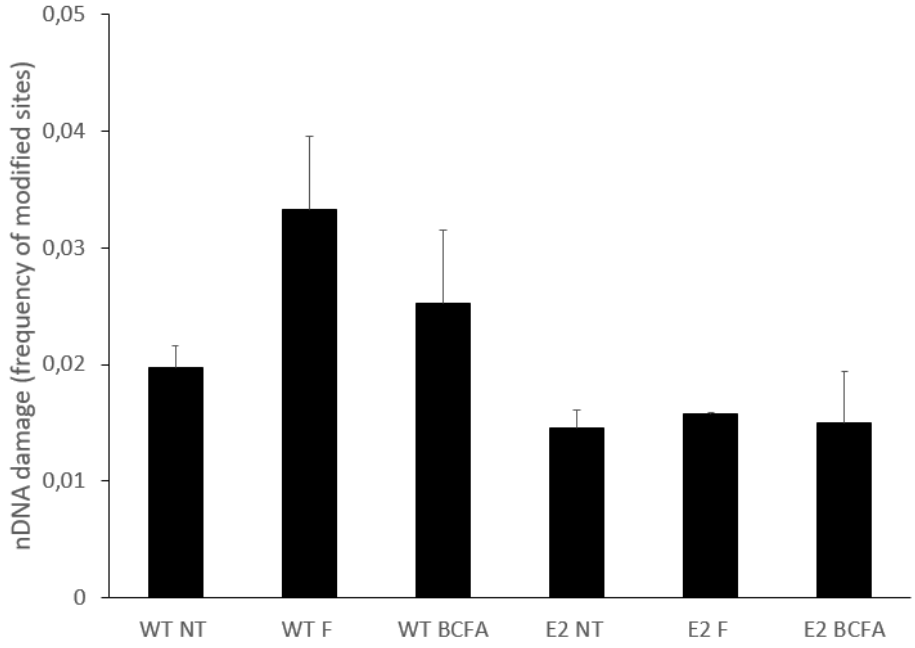


Figure 12. mtDNA and nDNA damage in HAP1 cells treated with fenofibrate and BCFA. HAP1 DNA damage was assessed by restriction enzyme inhibition as described in Methods and provided as the frequency which TaqI cuts DNA. mtDNA and nDNA damage from HAP1 cells treated with fenofibrate or BCFA for 1 day in cultivation medium at 37°C, before the medium was removed and the cells were harvested for DNA

damage analysis. A = mtDNA, B = nDNA (N=3) (All error bars are present, but the one for E2 F is too low to be visible in the figure).

The trends in Figure 12 implied that fenofibrate affected DNA damage differently in the two genotypes, and that BCFA reduced mtDNA damage. Additionally, nDNA damage in E2-deficient cells appeared to be unaffected by both fenofibrate and BCFA.

4.4.2 Investigating the effect of BCFA on BCAA/BCKA toxicity in repair-deficient MEFs

The relationship between BCAA/BCKA toxicity and DNA damage had been investigated in both OGG1-deficient MEFs and E2-deficient HAP1s. Therefore, as the effect of BCFA on BCAA/BCKA toxicity was studied in HAP1s, it was also natural to investigate if BCAA/BCKA toxicity in repair-deficient MEFs was altered by BCFA.

MEF survival was determined in samples co-treated with 5 and 10 mM BCAA/BCKA and 25 μM BCFA for 2 days. To discover if BCFA influenced cell growth, samples treated only with BCFA was compared to the control. BCFA affected cell growth in repair-deficient cells ($p<0.05$).

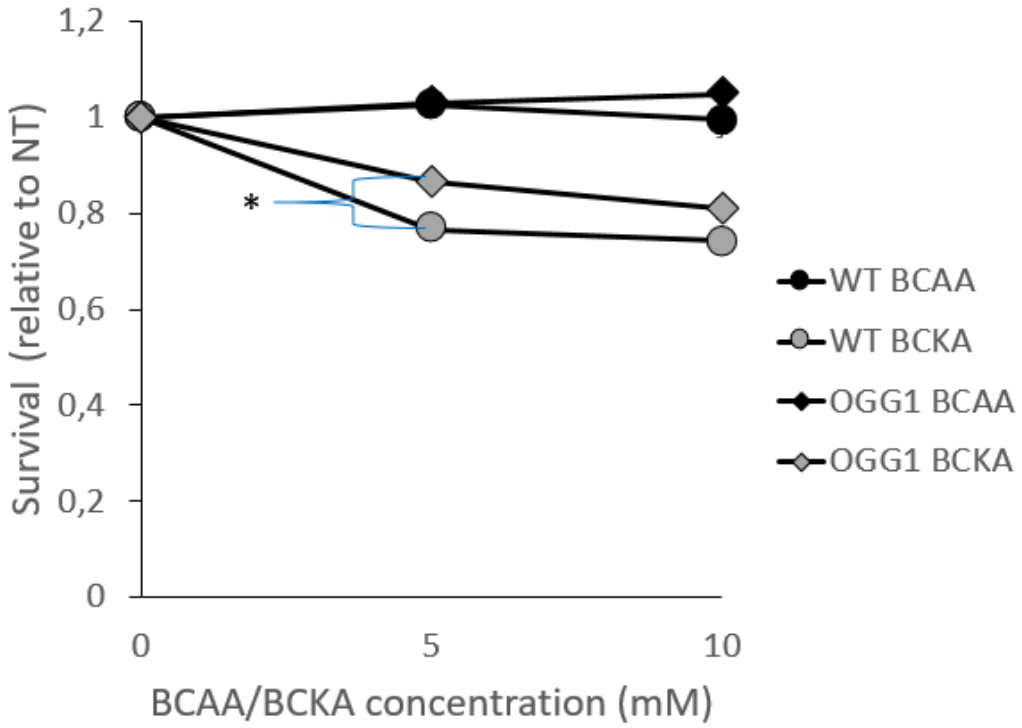


Figure 13. MEF sensitivity to BCAA and BCKA. MEF sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were incubated for 2 days with either BCAA or BCKA and BCFA in cultivation medium at 37°C for 2 days (N=3) (All error bars are present, but they are too low to be visible in the figure). The star (*) and the bracket indicate significant difference between WT and repair-deficient cells. * $p<0.05$.

Both MEF genotypes were significantly more sensitive to BCKA+BCFA than BCAA+BCFA ($p<0.01$) (Figure 13). 10 mM BCAA combined with BCFA increased survival of repair-deficient cells compared to the BCFA control ($p<0.05$), implying a stimulation of cell growth. The genotypes were equally affected by co-treatment with BCFA and BCAA at all concentrations. On the other hand, co-treatment with BCFA and 5 mM BCKA was still more toxic to WT than repair-deficient cells, but when the BCKA concentration increased they became equally affected.

As seen in comparison with Figure 9, BCFA appeared to restore sensitivity to BCKA in OGG1-deficient cells.

4.5 The effect of fenofibrate and BCFA on oxidative stress

4.5.1 Investigating if fenofibrate and BCFA protect HAP1s against oxidative stress

Although no connection between BCKA toxicity and DNA damage was found, other forms of damage caused by oxidative stress can induce cellular apoptosis in E2-deficient cells. As such, it was investigated if the E2 protein protect against oxidative stress, and if fenofibrate and BCFA increased survival of E2-deficient cells. The HAP1 cells were treated with menadione for 1 hour before they were grown for another 4 days. They were also pre-treated with either 25 μM fenofibrate or 25 μM BCFA for 1 day prior to menadione exposure.

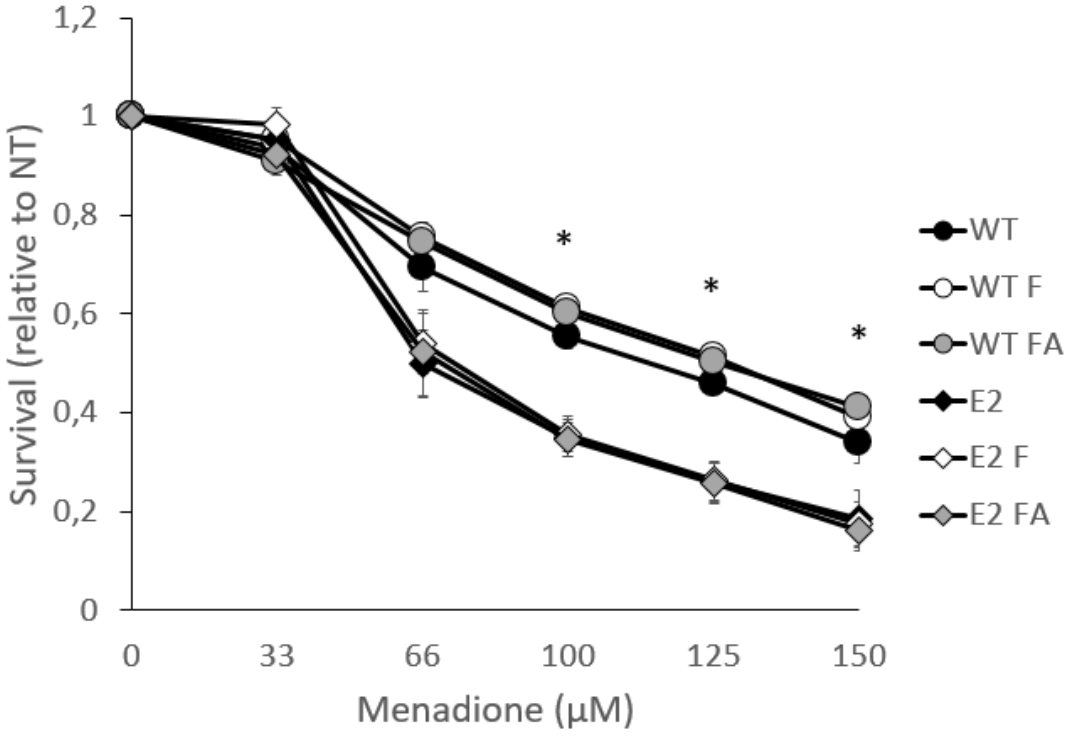


Figure 14. HAP1 sensitivity to menadione. HAP1 sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were exposed to menadione in cultivation medium at 37°C for 1 hour before the medium was replaced, and the cells were grown further for 4 days and analyzed. Pre-treatment with fenofibrate (F) or BCFA (FA) in cultivation medium at 37 °C lasted for 24 hours prior to menadione exposure (N=3) (All error bars are present, but some are too low to be visible in the figure). The stars (*) indicates significant difference between WT and E2-deficient cells, * $p<0.05$.

Figure 14 shows that WT and E2-deficient cell survival decreased with increased menadione concentrations. E2-deficient cells were significantly more sensitive than WT to menadione and menadione+fenofibrate at 100 and 125 μM, and significantly more sensitive than WT to menadione+BCFA at 100, 125 and 150 μM. Taken together, E2-deficient cells were more sensitive than WT, both with and without BCFA and fenofibrate present, when the menadione concentration increased. Neither BCFA nor fenofibrate increased survival of WT or E2-deficient cells, therefore they did not affect sensitivity to oxidative stress in HAP1s.

4.5.2 Investigating if fenofibrate alters menadione toxicity in MEFs

Finally, it was investigated if the OGG1 protein protect MEFs against oxidative stress, and if fenofibrate increased survival of repair-deficient cells. MEF cells were exposed to menadione for 1 hour before they were grown for another 2 days, and the MEFs were pre-treated with 25 μM fenofibrate for 1 day prior to menadione exposure.

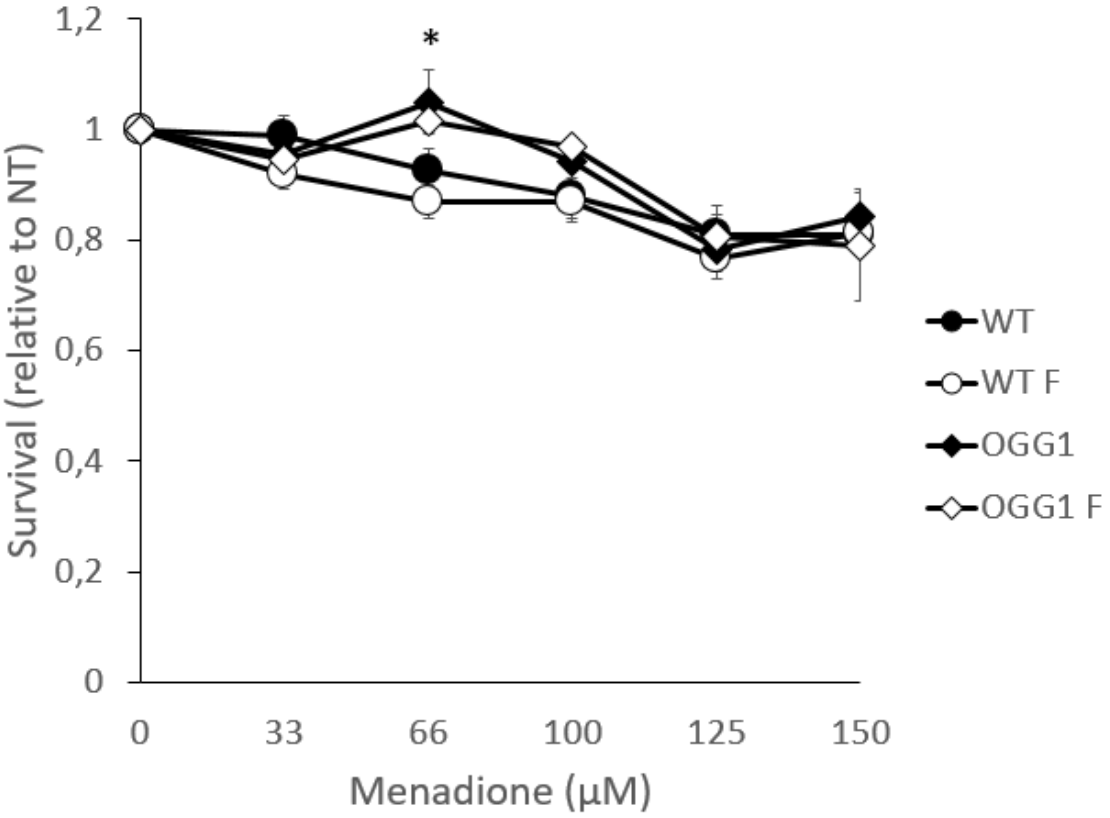


Figure 15. MEF sensitivity to menadione. MEF sensitivity was measured by the ability to reduce MTT as described in Methods. The cells were exposed to menadione in cultivation medium at 37°C for 1 hour before the medium was replaced, and the cells were grown further for 2 days and analyzed. Pre-treatment with fenofibrate (F) in cultivation medium at 37°C lasted for 24 hours prior to menadione exposure (N=3) (All error bars are present, but some are too low to be visible in the figure). The stars (*) indicates significant difference between fenofibrate-treated WT and repair-deficient cells, * p<0.05.

WT and repair-deficient MEFs reacted equally well to menadione-induced stress independent of the increased menadione concentration. Moreover, MEF survival was not affected by fenofibrate, except at 66 µM menadione (Figure 15), where OGG1-deficient cells displayed increased survival. This indicates that fenofibrate makes repair-deficient cells less sensitive to subtoxic levels of oxidative stress.

5 Discussion

5.1 BCAA/BCKA toxicity and DNA damage

In accordance with previous findings (Jouvet et al. 2000), this study confirmed that the BCKAs are more toxic than the BCAAs in MSUD (section 4.1, Figure 2; section 4.4.1, Figure 11). E2-deficient cells were used as model for MSUD, and therefore, it was expected that these cells should be more sensitive to BCKAs than to the WTs. To the contrary, WTs were significantly more sensitive than E2-deficient cells to 2 mM BCKA.

Although E2-deficient cells were shown to be more sensitive to 6 mM BCKA than the WTs, the WT BCKA survival curve flattens out after 2 mM BCKA, which might indicate a difference in saturation signal in the two genotypes. In the MTT assay, confluent cells (proliferating cells) as well as senescent cells reduce MTT (Berridge & Tan 1993; Edmondson et al. 1988). Senescent cells are cells that have stopped proliferating, but are still viable (Campisi & d'Adda di Fagagna 2007), and therefore, they are still capable of reducing MTT (Berridge & Tan 1993; Edmondson et al. 1988). Different initial proportions of senescent cells versus actively proliferating cells may have altered the interpretation if all the proliferating cells were growth arrested. At subtoxic levels, however, the putative contribution of non-replicative cells is smaller.

Interestingly, both fenofibrate and BCFA eliminated the significant difference between WT and E2-deficient cells at 2 mM BCKA (Figure 2 versus Figure 11). Additionally, Figure 11 indicates that BCKA was better tolerated in the presence of BCFA and fenofibrate ($p<0.01$) (60-80% survival vs. 50% survival). Thus, one interpretation is that BCFA and fenofibrate masks sensitivity to BCKA which somehow involves the E2 protein. There are 2 reasons which could explain that WT appears more sensitive to BCKAs than E2-deficient cells: 1) The E2 protein is needed to produce BCFAs, which may reach toxic levels. However, this is not in accordance with the beneficial effect of exogenously added BCFA. 2) E2 triggers conversion of BCKA to other types of toxic metabolites, but that upon treatment with BCFA and fenofibrate, the effect of the toxic metabolites is reduced. However, more and directly comparable experiments are needed to conclude on the details on the role of E2 in BCKA sensitivity, and the possibility of interference with BCFA and fenofibrate (see section 5.2.2).

The study aimed to investigate if DNA damage was the main cause of BCAA/BCKA toxicity in MSUD. The trends indicated that the BCKA toxicity was not attributed to increased DNA damage levels, and therefore, they could also indicate that mitochondrial dysfunction is not involved in BCKA toxicity. However, the correlation between mitochondrial dysfunction and DNA damage requires that mitochondrial dysfunction generates ROS. Reduced mitochondrial function would generate less ROS with consequential reduction of DNA damage, simply because of less ETC flux (Trifunovic et al. 2005). The tendency to increase DNA damage by fenofibrate (Figure 12, section 4.4.1) (which promotes mitochondrial efficiency) is an example of that ETC flux and DNA damage are associated processes, although not necessarily detrimental. Figure 3 indicated that BCKAs does not alter nDNA damage and reduces mtDNA damage, and that BCAAs increase nDNA damage, in WT and E2-deficient cells. Hence, the subcellular DNA damage pattern might also be altered.

The observed trends contradict previous studies on DNA damage and MSUD. Scaini et al. (2012) found that chronic BCAA treatment in an *in vivo* model for MSUD increased DNA damage in the striatum and hippocampus of rats. However, the authors could not be certain whether this effect was attributed to BCAAs or BCKAs, as BCAAs are degraded to BCKAs in the brain cells. Strand et al. (2014) found that patient fibroblasts had increased levels of mtDNA and nDNA damage, and that BCKA-exposed control fibroblasts accumulated similar amounts of mtDNA damage as the patient fibroblasts. They therefore concluded that BCKAs are DNA-damaging compounds, and that DNA damage might cause MSUD.

Furthermore, the sole effects of BCFA and fenofibrate on DNA damage was determined (Figure 12, section 4.4.1). Unfortunately, the DNA damage levels displayed much variation, which could be a result of decreased cell and mitochondrial quality over many cell passages (Hughes et al. 2007). However, it was interesting to note the trend where neither fenofibrate nor BCFA affected nDNA damage levels in E2-deficient cells, and that the DNA damage level was much higher in the mitochondria than in the nucleus. In the other DNA damage analyses, the mitochondrial and nuclear DNA damage levels were approximately the same.

Even if DNA damage cannot be confirmed as the cause of BCKA toxicity in E2-deficient cells, other forms of oxidative damage can occur due to increased oxidative stress. Many studies have reported increased oxidative stress through increased lipid peroxidation and reduced antioxidant defenses in MSUD. These features have been discovered in MSUD patients both at diagnosis and during treatment (Barschak et al. 2006; Barschak et al. 2008a;

Barschak et al. 2008b), and in *in vitro* studies on rats (Bridi et al. 2003; Bridi et al. 2005a; Fontella et al. 2002). Other studies have found that applying antioxidants in *in vivo* and *in vitro* models reduced oxidative DNA damage (Scaini et al. 2012), lipid peroxidation (Mescka et al. 2011) and morphological changes (Funchal et al. 2006), and all these findings indicates the presence of oxidative stress. In contrast to these findings, one study found reduced ROS content in fibroblasts from MSUD patients (Strand et al. 2014). However, this could potentially be a cell specific effect.

As oxidative stress has been frequently observed in MSUD, one might suspect the E2 protein of protecting cells from oxidative stress. In the present study, the E2 protein was shown to protect cells against oxidative stress as WT was less sensitive than E2-deficient cells (Figure 4, section 4.1). The E2 protein might protect the cells from oxidative stress itself, or perhaps some of the E2 products (e.g. CoA-derivatives) protect the cells. Another reason could be that having E2 stimulates and maintains other reactions and functions (e.g. respiration, mitochondrial function) which are important for cell survival.

The BCFAs applied did not protect cells against menadione-induced stress in either WT or E2-deficient cells, indicating that E2 products in the reaction pathway (e.g. other BCFAs) do not protect the cells. Furthermore, fenofibrate did not improve survival of WT or E2-deficient cells either, which indicates that it does not protect HAP1 cells against oxidative stress by improving mitochondrial function (Figure 14, section 4.5.1).

The repair-deficient MEFs were used as a model to evaluate the effect of DNA damage in cells. BCAA treatment did not alter sensitivity in MEFs, while BCKA treatment stimulated growth of repair-deficient cells. The MEF DNA damage trends (section 4.3, Figure 10) indicated that the BCKAs reduced mtDNA damage, but increased nDNA damage in repair-deficient cells. Hence, the DNA damage content does not correlate with stimulated survival of repair-deficient cells. BCFA reduced the significant difference between WT and repair-deficient cells treated with BCKA by restoring normal sensitivity in repair-deficient cells ($p<0.01$). As the BCKA concentration increased WT and repair-deficient cells became equally affected by BCKA (section 4.4.2). The results indicate that the sensitivity to BCKAs is associated with DNA repair through the transamination step, unless OGG1 performs another unknown function in the cells. Therefore, future studies should perhaps consider expressing OGG1 in OGG1-deficient cells to test if the cells imitate WT sensitivity. If they behave

similarly as WTs, OGG1 would be responsible for the BCKA sensitivity observed in the repair-deficient cells. Otherwise, a different DNA repair model (e.g. *Neil*$^{-/-}$) could be used to investigate if NEIL1-deficient cells react similarly as OGG1-deficient cells to BCAA and BCKA, and if BCFA reverses sensitivity to BCAAs and BCKAs.

Repair-deficient cells were equally sensitive as the WTs to menadione-induced stress, indicating that the OGG1 protein is negligible in the defense against oxidative stress (Figure 15, section 4.5.2). Fenofibrate increased survival of repair-deficient cells when the menadione concentration was sublethal (66 μM, Figure 15, section 4.5.2). Because these cells have a compromised DNA repair system, they should be more susceptible to oxidative DNA damage (Arai et al. 2002; de Souza-Pinto et al. 2001; Liu et al. 2011). As ROS frequently attack DNA, particularly mtDNA (Cui et al. 2012; Yu & Bennett 2014), one would have expected repair-deficient cells to be susceptible to increased oxidative stress. One possible explanation for the obtained results is that many MEFs have become senescent, and perhaps the cell features have been altered by accumulated mutations over many cell passages (Hughes et al. 2007), which perhaps has made the cells resistant to stress. Hence, the spectrophotometric measurement of reduced MTT is disturbed.

An interesting observation was that BCFA only affected growth in repair-deficient cells (section 4.4.2). BCFA treatment consisted of treating the cells with a stock containing 12-methyltetradecanoic acid and 15-methylpalmitic acid, and the first BCFA has been shown to stop proliferation in cancer cells (Yang et al. 2003). All the cell types used for this study have been immortalized (Horizon) (Klungland et al. 1999). Therefore, the 12-methyltetradecanoic acid should perhaps have stopped proliferation in all of them, but to the contrary, it is only growth of the repair-deficient cells that are reduced. However, as the repair-deficient cells lack the OGG1 protein, they should be more susceptible to mutations and DNA damage than the other cells (Arai et al. 2002; de Souza-Pinto et al. 2001; Liu et al. 2011). Mutations and DNA damage have close ties with cancer (Hoeijmakers 2009; Loft & Poulsen 1996; Valko et al. 2004), and therefore, lack of OGG1 might partly be responsible for the reduction observed in growth of these cells.

Fenofibrate's effect on mitochondrial function during oxidative stress was determined by respiration, gene expression and DNA damage analysis. Fenofibrate exerted positive effect on cells as it increased the respiration efficiency. However, a trend also indicated that fenofibrate

reduced the respiration capacity. Menadione, fenofibrate and co-treatment reduced expression of the chosen genes. As all the treatments resulted in down-regulated expression, the gene expression analysis indicated that fenofibrate and menadione must all function in the same metabolic pathways, but how reduced expression correlates with function must be investigated further in future studies. Moreover, the fact that fenofibrate significantly alters gene expression in most of the genes, implies that it is a transcription factor. One of the interesting observations was that fenofibrate reduced expression of *Ogg1*, which encodes the OGG1 protein of the BER pathway (de Souza-Pinto et al. 2001), but the cellular effect of down-regulating the expression of *Ogg1* must be determined in future studies. One study has found a correlation between reduced mitochondrial expression of *Ogg1* and mitochondrial dysfunction (Lee et al. 2013). Hence, fenofibrate might have reduced mitochondrial activity in the cells in the specific culturing conditions.

Menadione significantly induced DNA damage, and the trends implicated that fenofibrate induced DNA damage to a certain extent. This implies that menadione and fenofibrate has the same effect on the cells, although they are not equally toxic. However, the damage content of the co-treated sample was somewhere between the contents of the menadione and fenofibrate sample, which implies that fenofibrate might not have a DNA damaging effect. If fenofibrate induces DNA damage, the expected DNA damage levels in the co-treated sample would be higher than the one found in the menadione-treated cells. Thus, fenofibrate might reduce menadione-induced DNA damage, especially as the increase observed for the fenofibrate sample displayed more variation than the other samples, as shown in Figure 8 (section 4.2.2). Unlike the gene expression analysis, the DNA damage analysis indicates that menadione and fenofibrate do not operate in the same pathways because the co-treated sample display a DNA damage level between the fenofibrate and menadione sample.

Menadione treatment did not significantly alter respiration (Figure 5, Figure 6), but the trends observed in both figures indicates reduction of respiration. It would make sense that menadione affects respiration. Through being a ROS stimulant (Loor et al. 2010; Richter et al. 1995), it causes oxidative damage to lipids, proteins and DNA (Cui et al. 2012), and therefore, this treatment should affect respiration. After all, menadione seemed to be the main agent for the reduced respiration capacity in co-treated cells.

5.2 Procedure considerations

5.2.1 Cell-specific effects

The obtained results indicate that the E2 protein does not protect cells against BCKA toxicity. The outcome might have been caused by differences between HAP1 metabolism and metabolism in cells from MSUD patients. Additionally, HAP1 metabolism might also have caused the low DNA damage contents in BCKA-treated cells. Therefore, the HAP1 model must be analyzed further in future studies to discover the cause for WT being more sensitive than E2-deficient cells.

Ideally, cells from MSUD patients should be used because they would naturally be the best models for MSUD. The study completed by Strand et al. (2014) used fibroblasts from MSUD patients, and in contrast to this study, they found that BCKAs is more toxic than BCAAs, because they are the source of mtDNA damage in cells. However, these observations could be attributed to cell specific effects. Therefore, DNA damage analysis should be performed on several cell types from MSUD patients to investigate if the damage levels are similarly high in most tissues or if it is cell specific.

Several studies used brain or neuronal cells in their studies (Jouvet et al. 2000; Ribeiro et al. 2008; Scaini et al. 2012; Sgaravatti et al. 2003). Jouvet et al. (2000) argued that because the BCAAs and BCKAs accumulating in MSUD mainly affect white matter in the brain, they decided to use C6 rat cells. As MSUD symptoms include convulsions, coma, psychomotor delay and mental retardation (Sitta et al. 2014), the brain must be the main target for the accumulating BCAAs and BCKAs. Future studies should therefore perhaps consider using induced-pluripotent stem cells from MSUD patients.

Furthermore, as this study compares mouse and human DNA damage/repair systems, it is also subject to the limitations of comparing animal and human models. According to Wilson and Bohr (2007), comparing mouse and human DNA repair pathways have limitations due to important differences between the species' DNA repair pathways.

5.2.2 Cell treatments

The BCAA/BCKA concentrations used in these experiments are much higher than they are in MSUD cells from patients. Patients have concentrations below 1 mM (Strand et al. 2014). However, *in vitro* studies like this study, often use concentrations as high as 10 mM (Funchal et al. 2004; Strand et al. 2014).

Furthermore, long incubation periods were used to study HAP1 and MEF sensitivity towards BCAA/BCKA and menadione. This way, the cells could reach a density appropriate for spectrophotometric detection of reduced MTT, and therefore, it would be possible to correct for senescent cells and for cells that died immediately. Sometimes however, long incubations periods also seem to have allowed senescent cells to dominate the cell cultures (e.g. WT HAP1 sensitivity to BCKA in Figure 2, section 4.1). Hence, if they are not sensitive to treatment, they can potentially accumulate and thereby alter the spectrophotometric signal. Furthermore, the growth rate of the cells might have affected the analysis. For example, 10 000 MEFs might have reached 40 000 cells after 4 days, while 10 000 HAP1s might have reach only 25 000 cells after 4 days. Together, this means that the chosen incubation times can have masked differences between the genotypes and between treatments due to development of senescence and different growth rates of cells. For example, this might explain, why the WT MEFs display similar sensitivity to 5 and 10 mM BCKA and why reduction in survival of WT MEFs is quite small (section 4.3, Figure 9).

Next, the BCAA/BCKA sensitivity analyses and the BCAA/BCKA sensitivity analyses with BCFA and fenofibrate were performed at different days, both for HAP1s and MEFs. Therefore, the comparisons might be affected by differences in cell quality and treatment conditions between the two experiments, which means that the absolute values in the sensitivity figures cannot be compared. As such, if this study is repeated, the two experiments should be performed simultaneously.

5.2.3 Respiration and DNA damage analysis

In both the respiration analyses, menadione did not affect respiration. After the first respiration analysis, it was thought that menadione had no effect on the cells because the cell density was to high. However, when the cell number was reduced for the second respiration

analysis, menadione had no effect either. This could have been caused by that the menadione volume added to the cells was quite small compared to the solution in the dishes, and therefore, the number of cells that were affected by treatment might have been few. Additionally, if the fenofibrate and menadione treatments are compared, menadione had only 1 hour in the medium, while fenofibrate had about 24 hours, and therefore, menadione might not have had time to affect too many cells. One problem with reducing the cell density was that the oxygen consumption measurements became more unstable when the cell count was low (Miniaev et al. 2013).

Furthermore, prior to and after each respiration analysis, the oxygraph chambers must be cleaned with water and ethanol to remove cells and chemicals from the chambers (Gnaiger 2010). As the instrument had been in use for a long time, chemicals might have attached to the chamber walls, which might have disturbed the oxygen consumption measurements to a certain extent.

The DNA damage was detected with the TaqI inhibition method described by Wang et al. (2016). According to Wang et al. (2016), the method cannot be used to study clustered damage, and the method is affected by DNA fragmentation. Hence, as some forms of DNA damage are not detected, the damage analysis could potentially be affected. Therefore, this technique should perhaps be supplemented with a method which detects clustered damage, like the comet assay (Collins 2014; Shikazono et al. 2009). Scaini et al. (2012) found increased DNA damage in hippocampus and striatum an *in vivo* model for MSUD when applying the comet assay.

The DNA damage contents displayed much variation, and therefore, the trends must be verified in future studies. The high DNA damage variations observed might stem from that the DNA damage samples of the 3 parallels were not isolated simultaneously, which can have increased variations in the data (Wang et al. 2016).

5.3 Summary and future perspectives

The toxicity of BCKAs was not correlated to increased DNA damage levels. The E2 protein did not protect HAP1s against BCKAs, but provided protection against oxidative stress. BCFAs and fenofibrate made WT and E2-deficient cells equally sensitive to BCKAs, most

likely by generally increasing resistance to BCKAs. Neither BCFAs nor fenofibrate protected HAP1s against oxidative stress.

In future studies, this study should be repeated to validate the observed results and trends. The DNA damage analyses should be investigated further to find the source of the variation involved. Future studies should perhaps also consider using a mixture of BCAAs and BCKAs to assert their effect together, as this is the case in the MSUD patients, and they should investigate why BCKAs are more toxic than BCAAs. Moreover, they should investigate which of the BCKAs that is most toxic. The effect of BCAAs and BCKAs with and without BCFA and fenofibrate on HAP1 should also be analyzed with respiration, gene expression and DNA damage analyses. The E2-deficient cells as a model for MSUD should be further investigated to find out why the WTs were more sensitive than E2-deficient cells to BCKAs. Additionally, it should also be investigated why BCKAs appear to increase survival of repair-deficient cells, and why BCFA restores sensitivity to BCKAs. Finally, fenofibrate's effect on cells should be determined to evaluate if it has the same effect as menadione on cells.

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7 Appendix

Cell identity verification of MEFs

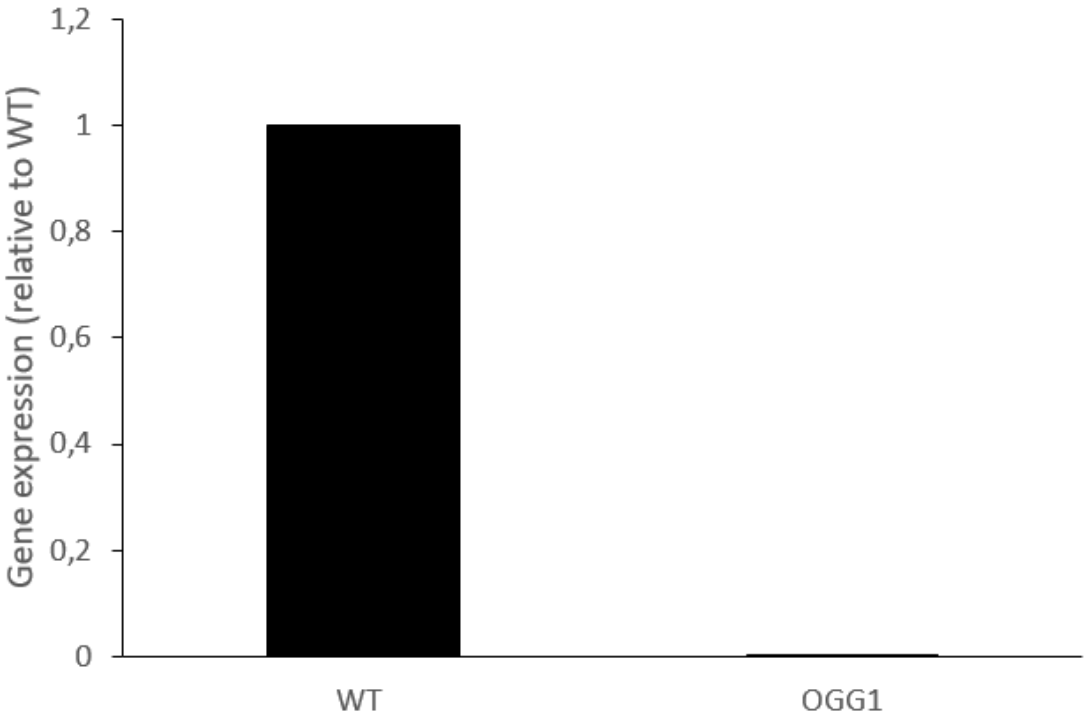
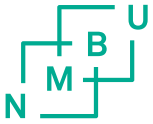


Figure A. Identification of MEF genotypes. WT and repair-deficient MEFs were harvested, and RNA isolation, cDNA preparation and gene expression analysis was performed as described in Methods (N=1). The analysis showed that the OGG1-deficient cells do not express *Ogg1*, in contrast to WT cells.



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