The influence of ketogenic therapy on the 5 R’s of radiobiology

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ABSTRACT

Purpose: Radiotherapy (RT) is a mainstay in the treatment of solid tumors and works by inducing free radical stress in tumor cells, leading to loss of reproductive integrity. The optimal treatment strategy has to consider damage to both tumor and normal cells and is determined by five factors known as the 5 R’s of radiobiology: Reoxygenation, DNA repair, radiosensitivity, redistribution in the cell cycle and repopulation. The aim of this review is (i) to present evidence that these 5 R’s are strongly influenced by cellular and whole-body metabolism that in turn can be modified through ketogenic therapy in form of ketogenic diets and short-term fasting and (ii) to stimulate new research into this field including some research questions deserving further study.

Conclusions: Preclinical and some preliminary clinical data support the hypothesis that ketogenic therapy could be utilized as a complementary treatment in order to improve the outcome after RT, both in terms of higher tumor control and in terms of lower normal tissue complication probability. The first effect relates to the metabolic shift from glycolysis toward mitochondrial metabolism that selectively increases ROS production and impairs ATP production in tumor cells. The second effect is based on the differential stress resistance phenomenon, which is achieved when glucose and growth factors are reduced and ketone bodies are elevated, reprogramming normal but not tumor cells from proliferation toward maintenance and stress resistance. Underlying both effects are metabolic differences between normal and tumor cells that ketogenic therapy seeks to exploit. Specifically, the recently discovered role of the ketone body β-hydroxybutyrate as an endogenous class-I histone deacetylase inhibitor suggests a dual role as a radioprotector of normal cells and a radiosensitizer of tumor cells that opens up exciting possibilities to employ ketogenic therapy as a cost-effective adjunct to radiotherapy against cancer.

Introduction

Within only one year after their discovery by Wilhelm Conrad Röntgen in his laboratory in Würzburg in 1895, X-rays were applied to treat patients with cancer by pioneers such as Victor Despeignes in Lyon (Sgantzos et al. 2014; Foray 2016) and — possibly — Emil Grubbé in Chicago (Grubbé 1933). In 1995, celebrating ‘100 years of Röntgen rays’, an editorial in the journal Strahlentherapie und Onkologie by E. Scherer concluded with the prospect that besides further technical refinements, the future of radiotherapy (RT) would mainly lie in its combination with chemotherapy, targeted therapies, hyperthermia, radiosensitizers and radioprotectors (Scherer 1995). While nowadays the majority of cancer patients in developed countries will receive RT as an essential part of their treatment (Miller et al. 2016), the search for good tolerable adjunct treatment options that could widen the therapeutic window remains a challenge. This is exemplified by the disappointing efficacy of personalized targeted therapies that seem to benefit at best a small percentage of patients, yet come with exorbitant costs and additional risks of severe side effects (Prasad 2016).

A completely opposite approach has been developed in the form of ketogenic therapy through calorie restriction (CR), fasting and ketogenic diets (KD) with the goal to exploit the metabolic differences between cancer and normal cells by altering the metabolic state of the cancer patient. Ketogenic therapy is of low costs, has comparatively minor side effects and simultaneously targets multiple signaling pathways in both tumor and normal cells with the potential to increase the therapeutic window when combined with other treatments such as radio- and chemotherapy (Lee & Longo 2011; Allen et al. 2014; Klement & Champ 2014; Woolf et al. 2016; O’Flanagan et al. 2017). As the name suggests, ketogenic therapy induces a physiological state of ketosis defined through elevated concentrations of the ketone bodies acetoacetate (AcAc) and β-hydroxybutyrate (BHB), the latter typically measuring >0.5 mmol/l. While long thought to simply serve as a backup-fuel during times of sparse carbohydrate consumption or starvation, recent findings have shown that ketone bodies exert pleiotropic effects as signaling molecules and epigenetic modulators, opening a wide variety of possible therapeutic applications (Newman & Verdin 2014; Rojas-Morales et al. 2016; Puchalska & Crawford 2017; Veech et al. 2017). In this review I, summarize the current evidence for beneficial effects when combining ketogenic therapy with RT with the aim to stimulate further research in this promising treatment approach.
Ketogenic therapy through fasting and ketogenic diets

Ketogenic therapy, also referred to as ketogenic metabolic therapy (Winter et al. 2017), is an umbrella term comprising CR, KDs, fasting and administration of exogenous ketone bodies. CR refers to diets restricting total energy intake without inducing malnutrition with % CR meaning that energy intake is restricted to (100-x)% of that normally consumed ad libitum whereby x is typically in the range of 20–50. Fasting is the most extreme form of CR (x = 100) and usually limited to a maximum of 3 days which is also referred to as short-term fasting (STF). KDs are isocaloric high-fat diets in which fat usually accounts for ≈75% of energy intake. Because the adaptations to fasting are mainly driven by the absence of carbohydrates (Klein & Wolfe 1992), KDs mimic major aspects of fasting including a decrease in insulin levels and activation of peroxisome proliferator-activated receptor (PPAR) pathways (Kurtak 2014) leading to an upregulation of fatty acid oxidation and an elevation of BHB and AcAc (Klement 2013, 2014; Klement & Fink 2016). Typical CR regimes in mice (≥30% CR over a few weeks) reduce glucose, insulin and insulin-like growth factor 1 (IGF-1) levels and elevate ketone body levels (Mahoney et al. 2006; Shelton et al. 2010; Jiang & Wang 2013; Morschher et al. 2015; Lashinger et al. 2016) to an extent which is comparable to humans on very low calorie diets or after STF (Mahoney et al. 2006). This review mostly focusses on STF and KDs, two metabolic therapies that have been shown to be feasible in their application to cancer patients in first pilot studies.

Tumor cell metabolism

Radiation oncologists are well aware of the value of FDG-PET (2-deoxy-2-[18F]fluoro-D-glucose positron emission tomography) for tumor imaging and radiotherapy (RT) treatment planning purposes. Regions of high FDG uptake are frequently boosted with higher doses simultaneously with the basic (planning target volume) dose application (Figure 1), and various measures of FDG uptake have been used to predict outcomes after RT (El Naqa 2014). It is important to recall that the utility of FDG-PET is based on a phenomenon systematically investigated nearly 100 years ago by Otto Warburg and coworkers (Warburg et al. 1924, 1926). Warburg measured a several-fold increased glucose uptake and lactate release of tumor tissue compared to normal tissue even in the presence of oxygen which is now referred to as the Warburg effect or aerobic glycolysis. He later proposed that damaged respiration would be the cause for this glycolytic phenotype (Warburg 1956). This “Warburg hypothesis” (which should not be confused with the Warburg effect) has gained support from detailed studies of tumor mitochondrial structure and metabolism (López-Rios et al. 2007; Hall et al. 2013; Gabriel et al. 2017).

Mitochondrial dysfunction in tumor cells has been related to morphological abnormalities, membrane lipid composition alterations, increased mitochondrial membrane potential and mutations in nuclear DNA (nDNA)- or mitochondrial DNA (mtDNA)-encoded genes that are part of the electron transport chain complexes; together these lead to respiratory insufficiency and increased production of mitochondrial reactive oxygen and nitrogen species (mtROS/mtRNS).
(Seyfried & Shelton 2010; Verschoor et al. 2013; Sullivan & Chandel 2014; Gaude & Frezza 2014; Seyfried 2015). Decreased ATP production through oxidative phosphorylation (OXPHOS) also leads to a compensatory increase in glycolysis by retrograde signaling involving stabilization of hypoxia-inducible factor 1α (HIF-1α) and subsequent upregulation of glycolytic enzymes and glucose transporters (Gillies et al. 2008; Seyfried & Shelton 2010).

Besides a compensatory ATP production mechanism (Zhuang et al. 2014), several benefits of increased glycolysis for tumor cells have been proposed, among them the possibility to survive in hypoxic environments and the production of ribose-5-phosphate and NADPH in the pentose phosphate pathway as a precursor and cofactor, respectively, for nucleic acid and lipid synthesis (Figure 1) (Gillies et al. 2008; Berardi & Fantin 2011). Most relevant to RT, however, is the possibility of tumor cells to attain antioxidative protection from glycolysis that involves NADPH and lactate and will be discussed further below. Ketogenic therapy tries to exploit this metabolic shift in tumor cells, which has the disadvantage of metabolic inflexibility concerning substrate utilization (Simone et al. 2013; Seyfried et al. 2014; Strowd et al. 2015; Lashinger et al. 2016; Klement 2017). In particular, many tumor cells appear to have lower expression of one or more of the enzymes needed to efficiently burn ketone bodies for ATP generation (Fredericks & Ramsey 1978; Tisdale & Brennan 1983; Skinner et al. 2009; Maurer et al. 2011; Chang et al. 2013; Morscher et al. 2015), although counterexamples exist (Schwartz et al. 2015). Holm and Kämerer (2011) reviewed data concerning substrate exchanges obtained intraoperatively on a total of 72 normal-weight colon, stomach and renal cell cancer patients. The data indicate almost no uptake of ketone bodies and free fatty acids by the tumors and in general a negligible exchange compared to glucose and lactate. In the study of Holm et al. (1995) measuring substrate balances across colon carcinomas, glucose uptake and lactate release of carcinomas were 30 and 43 times higher, respectively, than in peripheral tissues. Richtsmeier et al. (1987) reported larger percentagewise uptake of both ketone bodies in 10 head and neck cancer patients, but from their table 3 the absolute amount of BHB and AcAc consumed was only 0.09 mM and 0.07 mM, respectively, which was 19- and 25-fold less than that of glucose (1.75 mM). Finally, Kallinowski et al. (1988) measured substrate balances across breast cancer xenografts in rats and concluded that, while ketone body uptake was proportional to its supply, ‘… the overall contribution of ketone bodies to the energy status of breast cancer xenografts is probably minimal due to the small amount absorbed as compared with glucose’. None of the studies was able to provide clues whether ketone bodies were oxidized or not: ‘Further studies are required to determine if the ketone bodies are oxidized or contribute carbon to cell growth in human tumors’ (Richtsmeier et al. 1987). This issue is still uncertain today (Puchalska & Crawford 2017). Nevertheless, if ketone bodies are taken up by tumors but not oxidized, the possibility exists that they exert different functions, in particular their role as histone deacetylation (HDAC) inhibitors. This will be reviewed in the subsection on DNA repair below.

The effects of ionizing radiation: physics meets chemistry meets biology

While the primary physical interactions of modern-day clinical radiotherapy differ (mainly photo effect, Compton scattering and pair production at >1022 keV for γ-rays, Bremsstrahlung for electrons, Coulomb interactions for protons and other heavy charged particles (Marcu et al. 2012)), the further physicochemical interactions converge on the production of ROS, RNS and other free radicals, mainly from the radiolysis of cell water. Radiolysis of water yields H⁺, ·OH, hydrated electrons (e⁻aq), H₂ and H₂O₂. In the presence of oxygen H⁺ and e⁻aq rapidly react with oxygen and form the highly reactive superoxide radical O₂⁻, which greatly enhances the toxicity of RT (Azzam et al. 2012).

According to current textbooks nuclear nDNA is the crucial target of RT (Wouters & Begg 2009; Marcu et al. 2012). The potential of ionizing radiation (IR) to kill cells is greatly related to the fact that energy absorption occurs along distinct tracks with the potential to produce nDNA damage that is clustered within a few base pairs and much harder to repair than the randomly distributed lesions produced during every-day metabolic processes (reviewed in Lomax et al. 2013). This potential for inducing clustered nDNA damage is much greater for densely ionizing heavy charged particles than for sparsely ionizing electrons produced from X- and γ-rays. It is assumed that ~2/3 of nDNA lesions produced by γ-rays are due to indirect effects from free radicals produced in close vicinity to the DNA (Azzam et al. 2012). This already indicates that the amount of oxygen and anti-oxidants such as glutathione in the microenvironment play an important role in modifying nDNA damage.

Ionization clusters are also predicted to occur within mitochondria (Kam et al. 2013), with an impact on a cell’s fate that has so far probably been underestimated (Kam & Banati 2013; Richardson & Harper 2016). Compared to nDNA, mtDNA lacks histone protection and has less efficient repair mechanisms so that it is more vulnerable to IR (Yakes & Van Houten 1997; Larsen et al. 2005). Damage to mtDNA causes or augments mitochondrial dysfunction, increasing leakage from the electron transport chain with long-lasting increases in mtROS (in particular of O₂⁻) and mtRNS. Mitochondrial dysfunction triggers a retrograde stress response altering nuclear gene transcription (Cannino et al. 2007) that is principally able to account for the hallmarks of cancer and has been proposed as a therapeutic target for CR and/or KDs (Seyfried & Shelton 2010). Mitochondria are connected within clusters, and several studies have shown that this facilitates the propagation of damage responses to the nucleus. One mechanism involves mtROS/mtRNS-induced release of Ca²⁺ with subsequent uptake by adjacent mitochondria that in turn undergo a transient permeability transition with mtROS/mtRNS production and Ca²⁺ release, in this way propagating the signal (Leach et al. 2001). Direct and indirect effects of IR are also expected to damage the mitochondrial membrane structure through lipid peroxidation causing depolarization and mtROS release. A recent experiment using carbon ion and proton microbeams demonstrated that energy absorption within a mitochondrial cluster caused a near instant
findings that single-fraction stereotactic RT lacks a dose–response relationship and achieves lower tumor control rates ultimately reach the nucleus and induce ‘radiation-induced mitochondrial superoxide-mediated nuclear damage’. In line with this are findings showing that the mitochondrial location of antioxidative enzymes is more cell protective than the cytosolic; in particular, the expression of manganese superoxide dismutase (MnSOD) which dismutates $O_2^-$ to the less toxic $H_2O_2$ has been shown to be crucial for protection against IR-induced cell death (Kam & Banati 2013). Richardson and Harper (2016) argued that in oxygenated tumors and normal cells, IR is able to produce enough mtROS/mitRNS to account for the majority of nDNA damage and most of the observed RT effects.

Collectively these newer data reveal that while nDNA damage is the main determinant of RT-induced cell killing, this could be mainly a secondary effect of mtDNA damage and mtROS/mitRNS production. In addition, severe damage to mitochondria is able to trigger an increase in the mitochondrial membrane potential, leading to cytochrome c release and apoptosis (Ogura et al. 2009). Less severe damage can induce sustained mtROS production, retrograde signaling and long-lasting epigenetic nDNA modifications that contribute to late postradiation toxicity (Szumiel 2015). On the tumor cell level, both nuclear (Wouters & Begg 2009) and mitochondrial (Azzam et al. 2012) damage are thus able to induce a transient or permanent halt in the cell cycle or programmed cell death. On the whole tumor level, the outcome of RT will depend on five factors classically known as the 5 R’s of radiobiology that determine whether long-term tumor control will be achieved or not. Evidence that each of these factors can be modified through ketogenic therapy has been reviewed by us before (Klement & Champ 2014) and is strengthened by more recent data that will be discussed in the following section.

**Targeting the 5 R’s of radiobiology through ketogenic therapy**

**Reoxygenation**

The oxygen enhancement ratio describes the enhancement of RT efficacy with increasing oxygen concentrations and can reach values between 2 and 3 when comparing normoxic cells (21% $O_2$) to severely hypoxic cells ($\leq 0.1% O_2$). Reoxygenation of hypoxic tumor areas is therefore one of the main reasons why RT is applied in a fractionated scheme. Reoxygenation has recently gained renewed attention due to findings that single-fraction stereotactic RT lacks a dose–response relationship and achieves lower tumor control rates than multitreatment stereotactic RT even if the same biologically effective doses are applied (Guckenberger et al. 2013; Shuryak et al. 2015). This is consistent with a detrimental effect of missing reoxygenation in single-fraction RT (Lindblom et al. 2014). Among several strategies proposed to deliver oxygen to tumor cells, hyperbaric oxygen (HBO) before a RT session has shown some good clinical results in terms of improved local control rates and overall survival (Bennett et al. 2012; Stepieh et al. 2016). The group of Dominic D’Agostino has shown that HBO therapy increased ROS production and inhibited the growth of highly aggressive VM-M3 mouse tumor cells; its efficacy in vivo was thereby enhanced by simultaneously applying a KD and/or exogenous ketones (Poff et al. 2013, 2015). Thus, it could be speculated that HBO prior to a RT session can be made even more effective when the patient is in a state of ketosis. A recent case report of a poly-metastasized breast cancer patient in which a combination of HBO, KD, STF, glucose deprivation, hyperthermia and chemotherapy was used over 6 months reported a complete clinical, radiological and pathological response and provides a proof-of-principle example for such integrative treatment concepts (Lyikesici et al. 2017).

There is also evidence that ketogenic therapy alone could help to normalize the tumor vasculature and in this way facilitate oxygen diffusion to tumor cells. Selective inhibition of the vascular endothelial growth factor (VEGF)–VEGFR2 pathway in tumor cells through CR has been shown across several prostate and brain tumor models (Mukherjee et al. 1999, 2004; Powolny et al. 2008; Urits et al. 2012; Jiang & Wang 2013). In murine and human-derived brain tumors, CR upregulated $\alpha$-smooth muscle actin ($\alpha$-SMA, a marker for vascular smooth muscle cell-like pericytes) and suppressed VEGF and VEGFR2 expression, in this way reducing edemas and promoting vessel maturation (Urits et al. 2012; Jiang & Wang 2013). Similarly, in the GL26 murine glioma model, a KD significantly reduced edemas and decreased the protein expression of HIF-1$\alpha$ and VEGFR2. Although VEGF protein expression did not change, Vegfb gene expression was significantly reduced (Woolf et al. 2015). Since animals in the CR studies exhibited decreased IGF-1 levels, but a KD is not expected to decrease IGF-1 unless protein is also restricted (Klement & Fink 2016), this might explain the differences concerning VEGF protein expression changes, because IGF-1 is able to stimulate VEGF expression (Powolny et al. 2008). Collectively, these preclinical data have shown that decreasing blood glucose and elevating ketone body levels through ketogenic therapy could exert anti-angiogenic effects in brain and prostate tumors, in this way normalizing the tumor vasculature and principally allowing for facilitated oxygen delivery to tumor cells which would increase their radiosensitivity.

**DNA repair**

Chromatin structure is an important determinant not only of gene transcription, but also of DNA repair. Coordinated acetylation and deacetylation of histone lysine residues by histone acetyltransferases (HATs) and histone deacetylases (HDACs) is essential for efficient IR-induced double strand break (DSB) repair (Oike et al. 2014). Human HDACs are grouped into four classes according to their similarity to yeast enzymes, with classes I, II and IV being comprised of eleven Zn$^{2+}$-dependent HDACs and class III being comprised
of seven NAD\(^+\)-dependent sirtuins (Groselj et al. 2013). Ketogenic therapy is expected to increase the activity of sirtuins because it decreases glycolysis and promotes mitochondrial fatty acid oxidation, which is associated with an increase in NAD\(^+\) levels (Cantó & Auwerx 2012). There is evidence that SIRT1, a nuclear sirtuin which is activated through CR, KDs or fasting (Klement & Champ 2014), physically binds to and deacetylates the repair protein Ku70 after DNA damage, thereby enhancing the efficacy of nonhomologues end joining (NHEJ) DSB repair (Jeong et al. 2007). In line with this, Zhang et al. showed that SIRT1 inhibition impaired NHEJ repair in leukemia cells overexpressing SIRT1 through a mechanism involving increased acetylation of Ku70 (Zhang et al. 2016). On the other hand, there is data suggesting that sirtuin inhibition could improve NHEJ repair by facilitating the access of repair enzymes to damaged sites through histone hyperacetylation (Wojewodzka et al. 2007). These opposing effects thus could relate to the fact that sirtuins also target a variety of nonhistone proteins involved in the DNA damage response, so that the outcome of sirtuin inhibition strongly depends on cellular context (Kruszewski & Szumiel 2005).

More consistent data are available on inhibitors of class I and II HDACs that have shown potential to selectively radiosensitize tumor cells in vitro and in vivo by impairing both NHEJ and homologues recombination (HR) repair without harming, but possibly even benefitting normal cells (Groselj et al. 2013). The short-chain fatty acid butyrate, a class I and IIa HDAC differing from BHB by only a hydroxyl group, was shown to impair DNA DSB repair in melanoma cells, but not in normal human fibroblasts, in part by downregulating Ku70, Ku86 and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Munshi et al. 2005). The antitumor action of butyrate seems to depend on the presence of the Warburg effect (Donohoe et al. 2012) which probably also applies to BHB (Rodrigues et al. 2017). Donohoe et al. (2012) showed that butyrate can promote acetylation not only as a HDAC inhibitor but also as a HAT, especially at lower concentrations (0.5 mM). Concerning BHB, a recently published abstract reported that BHB radiosensitizes murine and human glioma and human glioma stem-like cells by altering key components of DNA damage repair through its action as a HDAC inhibitor (Woolf et al. 2017).

Since DNA repair is ATP dependent, ketogenic therapy could further compromise DNA repair in tumor cells that are dependent on glycolytic ATP production due to mitochondrial dysfunction. ATP deprivation of cancer cells through 2-deoxy-D-glucose, an inhibitor of glycolysis, or metformin, an inhibitor of respiratory chain complex I, has been shown to impair DSB repair (Jha & Pohlit 1992; Liu et al. 2012). In cells with inefficient respiratory function, glycolysis inhibition significantly decreased DNA DSB repair kinetics and radioreistance compared to those cells that were allowed to maintain high levels of glycolytic ATP production (Bhatt et al. 2015). Qin et al. (2015) revealed that cyclin-dependent kinase I relocates to mitochondria upon IR to increase complex I activity and ATP generation in order to sustain nDNA repair. Notably, increased complex I and IV activity in murine CT26 colon and 4T1 breast cancer cells was induced by STF in vitro and led to a subsequent boost in mtROS production and ATP depletion (Marini et al. 2016), providing further evidence that tumor cells with inefficient respiratory chain complexes would highly depend on glucose (and glutamine) fermentation in order to produce ATP for DNA repair.

Recently, Richardson and Harper (2016) proposed that oxygen-dependent ATP production through its relation to DNA repair efficacy constitutes one contribution to the oxygen enhancement ratio, the other one being the oxygen-dependent production of mtROS. In their explanation, as oxygen levels rise, damage from increasing levels of mtROS are more and more counter-acted by more efficient DNA repair due to increasing mitochondrial ATP production (Richardson & Harper 2016). However, the assumption of efficient oxygen-dependent ATP production in this model would not apply to those tumor cells that are unable to compensate for a loss of glycolytic ATP if forced to use mitochondrial metabolism in which case the overall oxygen enhancement ratio would be higher than predicted.

Finally, two studies investigated the combination of 30% CR and RT in triple negative breast cancer models and revealed synergistic antitumor effects that were related to a downregulation of the IGF-1 receptor (IGF-1R) and its downstream targets Akt and PI3K in both primary tumors and metastases (Saleh et al. 2013; Simone et al. 2016). IGF-1R overexpression in tumor cells is associated with high radioreistance due to the IGF-1R being involved in ATM (ataxia-telangiectasia mutated) mediated DNA DSB repair (Werner et al. 2016). These findings therefore suggest a possible role for ketogenic therapy as a targeted therapy against the IGF-1R pathway, providing another mechanism besides HDAC inhibition and ATP reduction for sensitizing tumor cells against IR by interfering with their DNA repair capacity.

### Intrinsic radiosensitivity/reactive species production

#### Increasing radiosensitivity of tumor cells

Radiosensitivity was introduced as the fifth R of radiobiology by Steel, McMillan and Peacock in 1989 (Steel et al. 1989). Alternatively, newer data would also support to choose ‘ROS/RNS production’ instead of (intrinsic) radiosensitivity, given the dominant role now attributed to mtROS/mTRNS production in the cellular response to IR (Kam & Banati 2013; Szumiel 2015; Richardson & Harper 2016). It is acknowledged that one important function of the Warburg effect in tumor cells is protection against intrinsically high levels of mtROS/mTRNS arising from mitochondrial dysfunction as mentioned above. One mechanism relates to high NADPH production during the oxidation of glucose-6-phosphate in the pentose phosphate pathway which maintains glutathione, the most important scavenger of H\(_2\)O\(_2\) and other peroxides, in the reduced state (Meister 1983). Another mechanism involves radical scavenging by lactate which is abundantly produced by Warburg-like tumor cells (Sattler & Mueller-Klieser 2009; Meijer et al. 2012). High tumor lactate concentrations have been directly linked to radioreistance in a variety of xenografted head and neck tumors that were irradiated using a clinically relevant schedule of 30 fractions over 6 weeks.
Impact of ketogenic therapy on tumor cells and normal cells during irradiation. Ionizing radiation leads to the formation of ROS, mainly from radiolysis of pool for H\textsubscript{2}O\textsubscript{2} scavenging. This is not the case in normal cells (B) which are able to efficiently burn fatty acids and ketone bodies in mitochondria which also optimizes the glutathione oxidant production. Inhibition of glycolysis by increases of ketone body and decreases in blood glucose levels depletes ATP and increases ROS production in tumor cells. This is not the case in normal cells (B) which are able to efficiently burn fatty acids and ketone bodies in mitochondria which also optimizes the glutathione pool for H\textsubscript{2}O\textsubscript{2} scavenging. The reduction of insulin (and IGF-1 in case of fasting) levels inhibits Akt signaling in normal cells, allowing FOXO transcription factors to translocate to the nucleus and promote a DNA repair and stress resistance program. Epigenetic modification through β-hydroxybutyrate also promotes FOXO3a, MnSOD and catalase transcription. This stress resistance program is ineffective in tumor cells with oncogene gain of function (e.g. IGF-1 receptor, PI3K) or tumor suppressor loss of function (e.g. p53, PTEN) mutations leading to an activation of the PI3K-Akt and other proliferation pathways and inactivation of FOXOs in the cytosol.

(Quennet et al. 2006; Sattler et al. 2010). Observations of decreased tumor lactate production in mice kept on a KD (Husain et al. 2014; Otto et al. 2014), cultured cancer cells treated with ketone bodies (Shukla et al. 2014; Kadocchi et al. 2017) or fasting-mimicking conditions (Bianchi et al. 2015; Marini et al. 2016) and importantly also cancer patients on a KD (Schroeder et al. 2013) could therefore build a basis to hypothesize that ketogenic therapy diminishes the antioxidative defense of tumor cells, forces them towards mitochondrial metabolism and through these two effects sensitizes them to IR or chemotherapy (Figure 2).

Indeed, much support for this hypothesis has been collected in recent years. First of all, glucose withdrawal was shown to lead to mtROS-mediated cell death in tumor cells, but not normal cells with intact mitochondria (Spitz et al. 2000; Ahmad et al. 2005; Jelluma et al. 2006; Aykin-Burns et al. 2009; Graham et al. 2012). Second, it has been shown that forcing Warburg-like tumor cells through STF to shift their metabolism from glycolysis and glutaminolysis toward the mitochondria resulted in enhanced oxygen consumption rate, high mtROS production and ATP depletion (Lee et al. 2012; Bianchi et al. 2015; Marini et al. 2016). Third, as shown in Table 1, several in vivo studies have reported synergistic antitumor effects between ketogenic therapy and various forms of chemotherapy, although some reported no such effects (Raffaghello et al. 2008; Safdie et al. 2012; Lee et al. 2012; Bianchi et al. 2015; D’Aronzo et al. 2015; Huisman et al. 2015; Huisman et al. 2016; Morscher et al. 2016; Pietrocola et al. 2016). Fourth, and in contrast to these latter studies, all studies published so far combining ketogenic therapy with RT have reported synergistic effects (Table 2) (Abdelwahab et al. 2012; Safdie et al. 2012; Allen et al. 2013; Saleh et al. 2013; Simone et al. 2016; Zahra et al. 2017). Collectively, these data support the hypothesis that KDs and STF are able to abolish the antioxidative defense and/or further increase mtROS/mtrNS levels in several tumor cell lines, sensitizing them to treatment by chemotherapy and RT.

Besides glycolysis, a second important adaption to high mtROS production frequently occurring in cancer cells is uncoupling of OXPHOS and ATP production through over-expression of uncoupling protein 2 (UCP2). This allows protons to leak from the intermembrane space back into the matrix, decreases the mitochondrial membrane potential and thus reduces the emission of mtROS (Mailloux & Harper 2011). UCP2 overexpression is considered a mechanism of RT and chemotherapy resistance and also has a metabolic action by supporting glucose and glutamine fermentation at the expense of mitochondrial oxidation (Vozza et al. 2014). However, this implicates inefficient mitochondrial ATP generation. Fine and colleagues have shown that UCP2 overexpression can be exploited therapeutically through administration of AcAc which led to ATP depletion and growth inhibition (Fine et al. 2009). They generally proposed that an ‘inefficient’ Randle cycle takes place in cancer cells in which glycolysis gets inhibited through free fatty acids and ketone bodies, but the cells would be unable to compensate for the reduced glycolytic ATP production due to uncoupling or general mitochondrial dysfunction.

**Increasing radioresistance of normal cells**

It has been shown that downregulation of the RAS-RAF-MAPK und Akt-mTOR signaling pathways that occurs in normal cells during STF is protective against the side effects of chemotherapy (Raffaghello et al. 2008; Lee et al. 2010). In contrast, tumor cells in which these pathways are

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**Figure 2.** Impact of ketogenic therapy on tumor cells and normal cells during irradiation. Ionizing radiation leads to the formation of ROS, mainly from radiolysis of cell water. ROS produced in the vicinity of DNA as well as long-lived ROS produced in mitochondria (H\textsubscript{2}O\textsubscript{2}) are able to diffuse to nuclear DNA and cause DNA lesions. Tumor cells (A) exhibit higher intrinsic ROS levels than normal cells due to mitochondrial dysfunction, which makes them dependent on glycolysis for anti-oxidant production. Inhibition of glycolysis by increases of ketone body and decreases in blood glucose levels depletes ATP and increases ROS production in tumor cells. This is not the case in normal cells (B) which are able to efficiently burn fatty acids and ketone bodies in mitochondria which also optimizes the glutathione pool for scavenging. This is not the case in normal cells (B) which are able to efficiently burn fatty acids and ketone bodies in mitochondria which also optimizes the glutathione pool for scavenging. The reduction of insulin (and IGF-1 in case of fasting) levels inhibits Akt signaling in normal cells, allowing FOXO transcription factors to translocate to the nucleus and promote a DNA repair and stress resistance program. Epigenetic modification through β-hydroxybutyrate also promotes FOXO3a, MnSOD and catalase transcription. This stress resistance program is ineffective in tumor cells with oncogene gain of function (e.g. IGF-1 receptor, PI3K) or tumor suppressor loss of function (e.g. p53, PTEN) mutations leading to an activation of the PI3K-Akt and other proliferation pathways and inactivation of FOXOs in the cytosol.
### Table 1. Studies adding fasting or a KD to chemotherapy.

<table>
<thead>
<tr>
<th>Study</th>
<th>Mouse strain</th>
<th>Tumor model</th>
<th>Intervention</th>
<th>Chemotherapy</th>
<th>Cumulative Dose (mg/kg)</th>
<th>Response measure</th>
<th>Fasting/KD induces a better response to chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffaghello et al. (2008)</td>
<td>A/J</td>
<td>NSX2 neuroblastoma i.v.</td>
<td>48 h STF</td>
<td>Etoposide</td>
<td>80</td>
<td>Mouse survival</td>
<td>No</td>
</tr>
<tr>
<td>Lee et al. (2012)</td>
<td>Female BALB/c, female and male C57BL/6 and nude mice</td>
<td>4T1 breast cancer, B16 melanoma, GL26 glioma, MDA-MB-231 breast cancer, OVCAR3 ovarian cancer, all s.c.</td>
<td>48–60 h</td>
<td>Cyclophosphamide (4T1), doxorubicin (all others)</td>
<td>150; doxorubicin: 10-40</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
<tr>
<td>Safdie et al. (2012)</td>
<td>Male C57BL/6 mice</td>
<td>GL26 glioma s.c.</td>
<td>48 h STF</td>
<td>Temozolomide</td>
<td>30</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
<tr>
<td>Bianchi et al. (2015)</td>
<td>Female BALB/c mice</td>
<td>CT26 colorectal tumor s.c.</td>
<td>48 h STF</td>
<td>Oxaliplatin</td>
<td>20</td>
<td>Tumor volume and glucose consumption from FDG-PET</td>
<td>Yes</td>
</tr>
<tr>
<td>D’Aronzo et al. (2015)</td>
<td>Female Nu/Nu mice</td>
<td>BxPC-3-luc pancreatic cancer s.c.</td>
<td>24 h STF</td>
<td>Gemcitabine</td>
<td>100</td>
<td>Tumor bioluminescence signal and tumor weight</td>
<td>Yes</td>
</tr>
<tr>
<td>Huisman et al. (2015)</td>
<td>Male and female FabpICre; Apc&lt;cre&lt;sup&gt;+&lt;/sup&gt; with a C57BL/6 background</td>
<td>Spontaneous intestinal tumors</td>
<td>66 h STF</td>
<td>Irinotecan</td>
<td>400</td>
<td>Tumor number and size</td>
<td>No</td>
</tr>
<tr>
<td>Huisman et al. (2016)</td>
<td>Male BALB/c</td>
<td>C26 colon carcinoma s.c.</td>
<td>72 h STF</td>
<td>Irinotecan</td>
<td>400</td>
<td>Tumor weight</td>
<td>No</td>
</tr>
<tr>
<td>Morschler et al. (2016)</td>
<td>Female CD-1 nude mouse</td>
<td>SH-SY5Y (TP53 wild type, non-MYC-N-amplified) and SK-N-BE2 (TP53 mutated, MYCN-amplified) neuroblastoma</td>
<td>4:1 KD 34/C2 1.8 Gy</td>
<td>Cyclophosphamide</td>
<td>1440&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Tumor volume and mouse survival</td>
<td>Yes</td>
</tr>
<tr>
<td>Pietrocola et al. (2016)</td>
<td>Wild-type C57BL/6 and athymic mice (nu/nu)</td>
<td>MCA205 murine fibrosarcoma, s.c.</td>
<td>48 h STF</td>
<td>Mitoxantrone, oxaliplatin</td>
<td>5.17, 10</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
</tbody>
</table>

CR: Calorie restriction; KD: Ketogenic diet; STF: Short-term fasting; i.v.: intravenously injected; s.c.: subcutaneously implanted.

<sup>a</sup>40 mg/kg/day over 36 days.

### Table 2. Studies combining ketogenic therapy with RT.

<table>
<thead>
<tr>
<th>Study</th>
<th>Mouse strain</th>
<th>Tumor model</th>
<th>Intervention</th>
<th>Fractionation</th>
<th>RT duration (days)</th>
<th>Response measure</th>
<th>Fasting/KD induces a better response to RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdelwahab et al. (2012)</td>
<td>Albino C57BL/6</td>
<td>GL26-luc2 glioma i.c.</td>
<td>4:1 KD</td>
<td>2 × 4 Gy</td>
<td>2</td>
<td>Tumor bioluminescence signal and mouse survival</td>
<td>Yes</td>
</tr>
<tr>
<td>Safdie et al. (2012)</td>
<td>Male C57BL/6N</td>
<td>GL26 glioma s.c.</td>
<td>48 h STF</td>
<td>5 + 2.5 Gy</td>
<td>7</td>
<td>Tumor volume and mouse survival</td>
<td>Yes</td>
</tr>
<tr>
<td>Allen et al. (2013)</td>
<td>Female athymic-nu/nu</td>
<td>H292 lung cancer s.c.</td>
<td>4:1 KD</td>
<td>6 × 2 Gy</td>
<td>10</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
<tr>
<td>Saleh et al. (2013)</td>
<td>Female BALB/c</td>
<td>H292 and A549 s.c.</td>
<td>4:1 KD</td>
<td>34 × 1.8 Gy</td>
<td>77</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
<tr>
<td>Simone et al. (2016)</td>
<td>Female BALB/c</td>
<td>67NR breast cancer o.i.</td>
<td>4:1 KD</td>
<td>2 × 6 Gy</td>
<td>2</td>
<td>Tumor size</td>
<td>Yes</td>
</tr>
<tr>
<td>Zahra et al. (2017)</td>
<td>Female athymic-nu/nu</td>
<td>MIA PaCa-2 pancreatic cancer s.c.</td>
<td>4:1 KD</td>
<td>6 × 2 Gy</td>
<td>10</td>
<td>Tumor volume and mouse survival</td>
<td>Yes</td>
</tr>
</tbody>
</table>

o.i.: orthotopically implanted; s.c. subcutaneously implanted.
constitutively activated through gain-of-function in oncogenes or loss-of-function of tumor suppressors would not benefit from this protection (Lee & Longo 2011). Hence, the term differential stress resistance has been coined (Raffaghello et al. 2008).

The fasting-induced reduction of growth factors such as glucose, insulin and IGF-1 inhibits RAS and Akt signaling, promotes adipose-free fatty acid release and hepatic ketogenesis and globally activates a stress resistance programme involving the activation of adenosine monophosphate-activated protein kinase (AMPK), PPARs and forhead box class O (FOXO) transcription factors (reviewed in Lee & Longo 2011; Kurtak 2014; Kopeina et al. 2017). PPARs, ‘the nuclear transcription factors of fat and fasting’ (Kurtak 2014), besides numerous metabolic actions, exert anti-inflammatory effects (Cullingford 2004; Kurtak 2014) and act as tumor suppressors during early stages of carcinogenesis by antagonizing Akt phosphorylation and promoting differentiation (Dong 2013). FOXOs are ‘survival transcription factors’ that inter alia promote the transcription of a number of DNA repair, cell-cycle arrest and antioxidant genes (Eijkelenboom & Burgering 2013). FOXOs are negatively regulated by phosphorylation through Akt which prevents their translocation to the nucleus and keeps them in the cytosol where they are prone to degradation by MDM2-induced polyubiquitination. In contrast, phosphorylation by AMPK activates FOXOs without directly regulating their localization (Eijkelenboom & Burgering 2013).

Ketone bodies have a possible role as radioprotectors due to their ability to increase the anti-oxidative defense mechanisms in cells, particularly in mitochondria. First, through its property as a HDAC class I inhibitor (specifically HDAC1 and HDAC2), BHB upregulates FOXO3a, thereby promoting expression of the anti-oxidative enzymes MnSOD and catalase (Shimazu et al. 2013; Nagao et al. 2016; Kong et al. 2017). Importantly, decreased HDAC1-3 protein levels and elevated MnSOD and catalase levels were measured in spinal cord tissue of KD-fed rats after spinal cord injury (Kong et al. 2017). Second, BHB was also shown to increase FOXO3a activity through direct AMPK phosphorylation (Bae et al. 2016). Third, BHB could mitigate extra-mitochondrial ROS generation by suppressing NADPH oxidase (NOX) expression that was shown to be due to selective HDAC1 and HDAC2 inhibition by BHB in PC12 cells (Kong et al. 2017). Fourth, the catabolism of ketone bodies in the Krebs cycle minimizes the cytosolic [NADP⁺/NADPH] redox potential which is the main determinant of the glutathione redox state, and therefore H₂O₂ destruction (reviewed in Veech 2004, 2017). Finally, the body may selectively utilize BHB as an endogenous protector against ROS as has been shown in the hearts of pressure-overloaded mice (Nagao et al. 2016) and recently suggested as a possible explanation for elevated serum BHB levels in head and neck cancer patients after RT (although reduced food intake as another possible explanation was not discussed; see Roś-Mazurczyk et al. 2017).

All of the preclinical studies summarized in Table 1 have confirmed reductions of side effects from a variety of chemotherapeutic drugs by ketogenic therapy without interfering with, or even boosting, these drugs’ antitumor effects. In addition, three small clinical studies have found first evidence for a protective effect of STF against chemotherapy-related cytotoxicity in humans (Safdie et al. 2009; de Groot et al. 2015; Dorff et al. 2016). Thereby, an important role for ketone body-mediated protection was obtained from the Dorff et al. study in which longer fasting duration prior to chemotherapy appeared more protective, yet only BHB, but not insulin, IGF-1 or glucose, were significantly different between 24h and 48h fasting prior to chemotherapy (Dorff et al. 2016). Collectively the data imply a possible role for ketone bodies, especially BHB, as radioprotectors, similar to what has recently been shown for oral administration of pynylbutyrate (Miller et al. 2017).

Redistribution

An integral part of the DNA damage response is the halt of cells in the G1, S or G2 phases of the cell cycle in order to allow DNA repair before progressing further or prepare for cell death in case of severe damage. ATM is the master regulator of these checkpoints by phosphorylating numerous downstream proteins some of which are inhibited (such as MDM2) while the majority are activated (such as p53 or BRCA1) (Pawlik & Keyomarsi 2004). Also, cells exhibit different cycle-dependent radiosensitivities, being most vulnerable to IR during the late G2 and M phase, less vulnerable during G1 and most resistant during the late S phase. Based on these concepts, fractionated RT aims at redistributing and arresting tumor cells at radiosensitive phases of the cell cycle, i.e. mainly G2/M (Pawlik & Keyomarsi 2004). Studies investigating the combination of STF with chemotherapy imply that normal cells interrupt their cell cycling and increase resistance against ROS by mechanisms involving Akt-mTOR inhibition, BHB elevation and FOXO3a activation (Lee & Longo 2011; Veech et al. 2017), suggesting a possibility for using ketogenic therapy for supporting the action of FOXOs in the DNA damage response (Eijkelenboom & Burgering 2013). In tumor cells, however, cell cycle checkpoints are often overridden by oncogene activation or loss-of- function of downstream ATM targets (Liang & Slingerland 2003). These differences could be exploited therapeutically by metabolic targeting. For example, p53-mutated cells have a deficient G1/S checkpoint control and accumulate at the G2/M checkpoint after IR whose initiation is not p53-dependent, although its duration is. These cells are very sensitive to a further shortening or abrogation of the G2 arrest (Strunz et al. 2002). It seems that STF has the ability to speed up the transition into mitosis in some oncogene-activated cells by further increasing phosphorylation of Akt, sensitizing them against DNA damage (Lee et al. 2012). In vitro cotreatment of cells with metformin and/or rapamycin with the mitotic inhibitors nocodazole or paclitaxel under STF mimicking low glucose conditions selectively killed p53-deficient tumor cells but protected normal cells which responded by undergoing G1 and G2 arrest (Apontes et al. 2011). But also p53 competent tumor cells may be sensitized to IR by ketogenic therapies in part by modulating their cell cycle as suggested by the molecular events induced in A549 lung cancer cells by metformin treatment (Storozhuk et al. 2013). Metformin exhibited radiosensitizing effects that were related to activation of ATM-AMPK-p53
signaling, inhibition of the Akt-mTOR pathway and a shift in the checkpoint activation pattern from S and G2/M toward G1. Notably, the same cell line used in a xenograft model showed a higher response to various RT fractionation schemes when the tumor-bearing mice were fed a KD, although cell cycle kinetics were not assessed (Allen et al. 2013).

Repopulation

In curative RT, achieving loss of reproductive potential of clonogenic tumor cells is at least as important as inducing apoptosis (Steel 2001). Clonogenic tumor cell division between RT fractions leads to a repopulation of the tumor and more cells that need to be killed. Tumors can even respond to prolonged treatment times (≥3 weeks) by accelerated repopulation which can significantly decrease the tumor control probability and provides a rationale for using accelerated or hypofractionated schedules, in particular for head and neck and non–small lung cancers. Repopulation has been shown to relate to Ki-67 staining which measures the fraction of tumor cells in active phases of the cell cycle (G1, S, G2 and M) as well as reoxygenation kinetics (Petersen et al. 2003). This implicates an important role of nutrient supply in driving repopulation, the composition and delivery of which is changed through ketogenic therapy.

For example, Martuscello et al. (2016) showed that glucose restriction lowered Ki-67 expression, clonogenic frequency and proliferation rate in patient-derived gliomaspheres, which were further reduced with the addition of 4 mmol BHB. There are multiple preclinical studies showing the potential of CR or KDs to delay tumor growth without further therapy as shown in two meta-analyses (Lv et al. 2014; Klement et al. 2016). Some of these studies measuring the fraction of Ki-67 positive cells found it to be significantly decreased, indicating G0 or early G1 cell cycle arrest and loss of reproductive integrity (e.g. Shelton et al. 2010; Morscher et al. 2015). Saleh et al. (2013) measured a more pronounced Ki-67 reduction with the combination of CR and IR compared to either treatment alone. Finally, a study in gastrointestinal cancer patients using the thymidine labeling index as a proxy for de novo DNA synthesis in S phase measured a decrease in proliferation under a high fat diet and an increase with a high glucose diet, although patient numbers were too small to reach statistical significance (Rossi-Fanelli et al. 1991).

Conclusions

The data reviewed here support the notion that the 5 R’s of radiobiology are intimately connected to cellular metabolism and could be modulated by ketogenic therapy in order to improve the outcome after RT, both in terms of higher tumor control and in terms of lower normal tissue complication probability. The metabolic shift from glycolysis toward mitochondrial metabolism has been shown to selectively increase ROS production and impair ATP production in glycolysis-dependent tumor cells. In contrast, lowering glucose and growth factors and elevating ketone body concentrations causes normal cells to switch to a cellular maintenance and stress resistance program. First clinical studies confirming that a differential stress resistance can be induced by STF in humans have been published; the question; however, whether such resistance can be mimicked by KDs and in this way be utilized for a longer course of RT has not been clinically studied yet. Besides this, a number of research questions can be identified that should be addressed in future studies: (i) To what extent would exogenous BHB, alone or in combination with a KD or STF, act as a simultaneous radioprotector of normal tissue and a radiosensitizer of tumor cells, e.g. when applied prior to high-dose stereotactic RT? (ii) How would CR, STF or a KD affect the redistribution and synchronization of normal and cancerous cells during multi-fraction RT? In particular, what effect would refeeding after STF have on the cell-cycle distribution of normal cells in the next RT fraction? (iii) Is there any risk of unwittingly protecting some kinds of tumor cells through ketogenic therapy, or enhancing their proliferation, as suggested by some recent studies (Rodrigues et al. 2017; Xia et al. 2017)? Notably, one preclinical study suggested that a KD might drive tumor growth of BRAF-mutated melanoma (Xia et al. 2017), but it was exactly this tumor that responded best in a series of cancer patients undergoing a KD (Tan-Shalaby et al. 2016), pointing out a general difficulty with the validity of preclinical research findings for clinical reality. In the end, this cautious note also applies to most of the concepts developed and summarized here that ultimately need to be tested in clinical settings.

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