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**Title page:**

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**Abstract.**

Glioblastoma is the most frequent and malignant brain tumour. For many years, the conventional treatment has been maximal surgical resection followed by radiotherapy (RT), with a median survival time of less than 10 months. Previously, the use of adjuvant chemotherapy (given after RT) has failed to demonstrate a statistically significant survival advantage. Recently, a randomized phase III trial has confirmed the benefit of temozolomide (TMZ) and has defined a new standard of care for the treatment of patients with high-grade brain tumours. The results showed an increase of 2.5 months in median survival, and 16.1 % in 2 year survival, for patients receiving RT with TMZ compared with RT alone. It is not clear whether the major benefit of TMZ comes from either concomitant administration of TMZ with RT, or from six cycles of adjuvant TMZ, or both.

The objectives were to develop our original model, which addressed survival after RT, to construct a new module to assess the potential role of TMZ from clinical data, and to explore its synergistic contribution in addition to radiation. The model has been extended to include radiobiological parameters. The addition of the linear quadratic equation to describe cellular response to treatment has enabled us to quantify the effects of radiation and TMZ in radiobiological terms.

The results indicate that the model achieves an excellent fit to the clinical data, with the assumption that TMZ given concomitantly with RT synergistically increases radiosensitivity. The alternative, that the effect of TMZ is due only to direct cell killing, does not fit the clinical data so well. The addition of concomitant TMZ appears to

change the radiobiological parameters. This aspect of our results suggests possible treatment developments.

Our observations need further evaluations in real clinical trials, may suggest treatment strategies for new trials, and inform their design.

**Keywords.**

Glioblastoma, radiotherapy, temozolomide, Linear Quadratic model, dose escalation, tumour regrowth time.

**1 Introduction**

High grade gliomas are the commonest form of primary brain tumours. Glioblastoma (GBM), the major and most aggressive type of glioma, has a poor prognosis, with a median survival until recently ranging between 9 and 12 months (DeAngelis, 2001; Kleihues and Cavenee, 2000). Although primary tumours of the central nervous system (CNS) account for only 2 % of all primary tumours, about 4500 *per annum* in the UK, they are responsible for more loss of life per patient than any other adult cancer, at just over 20 years per patient (Burnet et al., 2005), because largely of glioblastoma.

Radiotherapy is a valuable post-operative treatment, with dose an important determinant of tumour control (Larson and Wara 1998; Walker et al., 1979; Werko et al., 1996).

However, little had changed in the treatment of this disease for the past 40 years until recently. Studies of combined radiotherapy and chemotherapy performed in the 1970s-1990s using nitrosourea-based chemotherapy given after radiotherapy showed equivocal

results. These chemotherapy regimens had only modest efficacy at best and appreciable toxicity; some studies showed no benefit (MRC Brain Tumour Working Party, 2001).

In 2005 Stupp et al. reported the early outcome of an international multi-centre randomised phase III trial conducted by the European Organisation for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) Clinical Trials Group (trial 26981-22981/CE.3). This trial involved 573 patients with newly diagnosed GBM that randomly received either radiotherapy only or the same radiotherapy plus concomitant temozolomide (TMZ), followed by six cycles of adjuvant TMZ. The outcome of this study demonstrated a statistically significant and clinically meaningful survival benefit in the addition of TMZ. The median survival was 12.1 months with radiotherapy alone and 14.6 months with radiotherapy plus TMZ, a gain of 2.5 months. More importantly, the two-year survival rate was 10.4 % in patients treated with radiotherapy alone and 26.5 % in patients assigned to radiotherapy plus TMZ. This result translates into a reduction in the risk of death of 37 % for patients treated with radiotherapy plus TMZ, as compared with those who received radiotherapy alone (Stupp et al., 2005). This has been corroborated in a separate, smaller randomised Phase II study (Athanassiou et al., 2005). However, patients continue to die after this point, and the 5-year survival rates of the EORTC-NCIC trial were 1.9 % with radiotherapy alone and 9.8 % for those treated with radiotherapy plus TMZ (Stupp et al., 2009).

TMZ is an oral alkylating agent, first synthesized in 1984 (Stevens et al., 1984) and approved in 1999 by the Food and Drug Administration (FDA) for the treatment of progressive or recurrent GBM. Preclinical studies established that TMZ crosses the blood-brain barrier (BBB) and is spontaneously converted to its active metabolite 5-(3-methyltriazene-1-yl)imidazole-4 carboximide (MTIC) within the tumour cells. This

metabolite is responsible for the interruption of DNA replication by methylation of the O<sup>6</sup> position on guanine. The O<sup>6</sup> methylguanine produced by TMZ, is incorrectly paired with thymine during subsequent rounds of replication, inducing cell death (Stevens et al., 1987). Its clinical use was developed during the 1990s (Newlands et al., 1997); direct cytotoxic effects were observed in patients with relapsed disease (Newlands et al., 1996).

The EORTC-NCIC trial used concomitant radiotherapy and chemotherapy, followed by six months of chemotherapy alone (adjuvant chemotherapy). This combined treatment demonstrated a statistically significant and clinically important improvement in survival. Although toxic effects and disease progression were reported during the adjuvant period, the reported survival advantage has been the first major advance for half a century.

There is a debate amongst clinicians as to whether the major benefit of TMZ comes from its effects with concurrent radiotherapy, or due to the six cycles of adjuvant treatment, or both (Nieder et al., 2009; Stupp et al., 2009; Villano et al., 2009). It is perhaps relevant that only 78 % of the TMZ-treated group started the adjuvant component of the chemotherapy following radiotherapy, and only 47 % of patients completed 6 cycles, the main reason for which being disease progression. This major clinical advance was therefore made without the benefit of adjuvant TMZ in many of the patients.

Given the clear efficacy of the combined treatment, including its adjuvant phase, it might now be difficult to perform a study comparing the effects of TMZ given during radiotherapy alone, with TMZ given only in the adjuvant setting, or with the

concomitant and adjuvant schedule. Thus mathematical modelling is suggested as a means whereby the potential interaction between TMZ and radiation can be explored. The identification of the real role of TMZ could then be employed to design future clinical trials using optimised combinations of treatments.

In this paper, after a short review of some recent studies that specifically attempt to describe high-grade brain tumour response to radiotherapy and chemotherapy, we briefly present the development of our previous mathematical model. This model was developed to extract biological information from clinical data of patients with GBM treated with radiotherapy alone, to demonstrate the adverse effect of delays in starting radiotherapy and to evaluate the potential value for radiotherapy dose escalation (Burnet et al., 2006; Kirkby, 2007a; Kirkby et al., 2007b).

In the present work, we first incorporated in the model the effects of chemotherapy to study the direct anti-tumour activity of TMZ. The interaction between TMZ and radiotherapy is likely to be due to a combination of mechanisms that include several biological pathways. In this paper we focus mainly on two extreme scenarios: Scenario 1, TMZ as a radiosensitizer, and Scenario 2, TMZ as a cytotoxic drug, independent of radiotherapy. The radiosensitization depends mainly on the potential of TMZ to sensitize tumour cells to radiotherapy, essentially decreasing the cell survival fraction in response to a single exposure of radiation. In contrast, for independent cytotoxicity, we mean that TMZ alone causes independent cell kill regardless of the radiation.

Another key aspect of this study is the incorporation of the Linear Quadratic (LQ) equation (Hall and Giaccia, 2006), in order to estimate the *in vivo* alpha-beta ratio of GBM tumour cells and the equivalent effect of TMZ in Biologically Effective Dose



(BED) units of radiotherapy. The LQ model is the most widely used model in radiotherapy (Fowler, 2006). It describes cell survival after exposure to ionizing radiation, taking into consideration two forms of DNA damage, expressed by the linear and the quadratic terms. This extension to our model enables us to quantify the variability of the radiosensitivity parameters for both arms of the EORTC-NCIC study and in particular the influence of TMZ on GBM cell radiosensitivity. This extension has an additional advantage because it can be used to test alternative dose fractionation schedules.

Finally, we present our preliminary results for both scenarios, fitting the model to half of the dataset of the EORTC-NCIC study, to identify the main effect of TMZ. In addition, we indirectly validate our results by comparing the equivalent radiobiological parameters extracted from both arms of the trial with previously published studies.

## **2 Previous studies**

There are a limited number of publications about modelling radiotherapy combined with chemotherapy in patients with GBM. The majority of the previous models have been developed to describe the separate effects of radiotherapy and chemotherapy at a single patient level.

The first modelling attempt of chemotherapy in glioma dates from 1995, when Traqui et al. (1995) tried to describe the effects of chemotherapy on the spatio-temporal growth of the tumour. Chemotherapy was assumed to be spatially homogeneous in the brain and its effects were mathematically approximated via a reaction-diffusion system. The authors finally assessed the model using a serial of computed tomography (CT) scans

from a patient with recurrent astrocytoma treated with two different chemotherapeutic agents.

Swanson et al. (2002) developed the Traqui model further, taking into consideration the brain structure differentiation into grey and white matter. They assumed that chemotherapy affected mainly those cells within the grey matter, whereas those in the white matter were still highly proliferative. This was explained by the variability in vascular density throughout the brain, which is well known to be higher in the grey matter. The results reported could explain the clinical problem of apparent tumour reduction in certain areas of the brain (below the detection limit of the imaging modalities) with unaffected growth in other regions.

Powathil et al. (2007) have tried to describe the effects of radiotherapy and TMZ, and to predict the optimal sequencing of their combination. They developed a spatio-temporal mathematical model based on reaction-diffusion equations. Radiotherapy was included using the LQ model and a cytotoxic chemotherapy effect was integrated via a log-kill model. They tested alternative radiotherapy and chemotherapy schedules: radiotherapy dose fractionation and combinations of neo-adjuvant (chemotherapy given alone prior to radiotherapy), concurrent and adjuvant TMZ. The results showed, in the hypothesis of pure TMZ cytotoxicity, that neo-adjuvant, then radiotherapy alone followed by adjuvant TMZ might be a better protocol than current clinical practice.

More recently, Eikenberry et al. (2009) developed a three-dimensional brain model for simulating surgical resection, radiotherapy and chemotherapy. They examined a clinical case of a patient with recurrent high-grade brain tumour who received TMZ-chemotherapy after the first resection and compared the effects with the model's

prediction. Initial results suggested that increasing resection margins significantly reduced post-operative tumour recurrence. Radiotherapy was predicted to be unsuccessful in hypoxic areas, and the irregular vasculature hampered TMZ activity.

To our knowledge, no attempt has been made to formulate a mathematical model of a population of patients with GBM treated with radiotherapy and chemotherapy. In the following we propose an extension of our previous mathematical model that may address this issue.

### **3 Clinical data**

A randomly selected half of the data set of the EORTC-NCIC trial 26981-22981/CE.3 was kindly released by Thierry Gorlia, EORTC. Clinical data were provided for 287 patients, randomly selected from a total of 573 patients who participated in the original randomised trial (Stupp et al., 2005). Patients in that study had to fulfil rigorous eligibility criteria, and within 6 weeks of biopsy or surgical resection were randomly allocated to receive radiotherapy alone (143/286 patients), or radiotherapy plus concomitant and adjuvant TMZ (144/287 patients). Radiotherapy consisted of a dose of 2 Gy per fraction given 5 days per week for 6 weeks. Concomitant chemotherapy consisted of oral TMZ ( $75 \text{ mg/m}^2$  per day), given daily from the first to the last day of radiotherapy including weekend days when radiotherapy was not given. Adjuvant chemotherapy comprised 6 cycles of TMZ given for 5 days every 28 days, with a dose of  $150 \text{ mg/m}^2$  for the first cycle, then  $200 \text{ mg/m}^2$  for 5 subsequent cycles. In the trial, the time delay between the first presentation to the oncology unit and the commencement of the treatment had a mean of 52.15 days, variance  $28 \text{ days}^2$  for the

radiotherapy only arm, and a mean of 43.4 days, variance 11 days<sup>2</sup> for the radiotherapy plus TMZ arm.

The survival data in the form of Kaplan-Meier survival curves (Kaplan and Meier, 1958) are shown in Fig. 1.

#### 4 Original model

This paper describes extensions of the original brain tumour model to include chemotherapy and radiobiology. A detailed description of this model is given in Kirkby et al. (2007b).

Briefly, the model is based on three main levels. At the first level, the single patient is modelled at a cellular scale using exponential growth for the tumour cells ( $C(t)$ ):

$$C(t) = C_0 \exp(k_c t) \quad (1)$$

where  $C_0$  is the number of cancer cells in the brain at presentation (cells) and  $k_c$  is the rate constant for cancer cell growth (days<sup>-1</sup>). The tumour indirectly causes normal cell ( $N(t)$ ) damage via an assumed reaction,  $N + C \xrightarrow{k_n} C$  (Kirkby et al., 2007b). Hence the number of normal brain cells with time is given by

$$N(t) = N_0 \exp\left(\frac{-k_n C_0}{k_c} (\exp(k_c t) - 1)\right) \quad (2)$$

where  $N_0$  is the number of normal brain cells left at presentation (cells) and  $k_n$  is the rate constant for elimination of normal cells (cells<sup>-1</sup> days<sup>-1</sup>). Death occurs when the normal brain cell number falls below a critical level ( $N_{crit}$ ) that is required for the patient to remain alive.

The treatment, which is responsible for the temporary arrest or complete destruction of the tumour volume, is represented by

$$C(t_{delay}^+) = C(t_{delay}^-)x_s^j \quad (3)$$

where  $t_{delay}$  represents the time delay to commence treatment from presentation (days),  $t_{delay}^+$  and  $t_{delay}^-$  represent respectively a time just after and before the radiation treatment (days),  $x_s$  is the survival fraction after a single exposure of radiation (dimensionless), and  $j$  is the number of fractions of radiotherapy. Two assumptions are made in Eq. (3), that all the radiation exposures are delivered at the same instant and that all the normal brain cells survive the treatment.

At the second level, Monte Carlo simulation is used to generate a population of patients for a virtual clinical trial. The model is then assessed by comparing the *in-silico* patient survival curve with the real clinical trial survival curve.

The main parameters for an individual patient are:

1. the number of normal brain cells at presentation  $N_0$ ,
2. the tumour doubling time  $t_D$ ,
3. the rate constant for damage to normal cells  $k_c$ ,
4. the delay to start treatment  $t_{delay}$ ,
5. the critical number of normal brain cells needed for the patient to remain alive  $N_{crit}$ , and
6. the surviving fraction of tumour cells after each fraction of radiotherapy  $x_s$ .

The values of these parameters are assumed to be randomly distributed, we vary the mean and variance of these distributions in order to most closely match the clinical data.

At the third level, the values of the unknown parameters in the probability distributions are determined by fitting to the real clinical survival data, by a combination of folding polygon and simulated annealing techniques (Kirkby et al., 2007b).

In addition, the model contains a mathematical representation of the patient eligibility criterion which delineates the patient target population to be included in a clinical trial. In effect, the computer can generate patients whose conditions are too ‘poorly’ to be eligible for the radical treatment. The model can estimate how long the patient would survive without treatment at the moment of presentation and start of the treatment. These values are then used as a basis for choosing what type of treatment to offer (e.g. radical or palliative treatment). Those patients who are already in poor condition and therefore would not benefit from radical treatment (where ‘radical’ is intended to mean ‘with curative intent’) are directly rejected by the model.

## **5 The chemo-radiotherapy model**

The EORTC-NCIC study described above implies that the study of the full sequence and scope of action of TMZ should be given high priority. TMZ is given in combination with radiotherapy and subsequently as adjuvant therapy. The relative contribution of the concomitant and adjuvant chemotherapy needs to be assessed before rational application, especially as the EORTC-NCIC trial design did not distinguished the relative contribution of the concomitant and adjuvant phases. Exploratory modelling may improve knowledge of the relative benefit of concomitant and adjuvant TMZ.

Both pharmacokinetic and pharmacodynamic aspects of the drug’s activity were

considered in the model. The short half-life (1.8 hours) and rapid absorption (maximum concentration achieved in 0.9 hours) suggested that no modelling of steady-state drug accumulation was necessary (Brada et al., 1999; Hammond et al., 1999). The chemopharmacodynamics were assumed to possess similar characteristics to those of radiation, such as the concepts of tumour cell kill and therapeutic gain (Steel, 2002).

In order to model chemoradiation therapy, we consider two different possible scenarios, that TMZ 1) may improve survival potentiating the local efficacy of radiotherapy, or 2) may have an independent effect directly killing the tumour cells.

### 5.1 Scenario 1: radiosensitization

TMZ delivered during radiotherapy could result in survival benefit due to TMZ-mediated radiosensitization. In this scenario we calculate the radiation enhancement of TMZ on the surviving fraction of cancer cells in response to each single exposure of radiation. This implies that the TMZ effect is associated with each dose of radiation such that a dose of 2 Gy together with the drug kills a larger number of cells than a 2 Gy dose of radiation alone.

It is now appropriate to reiterate two important equations. Firstly, the treatment model equation (Eq. (3)) is slightly changed from the original model. The previous model assumed that radiotherapy was applied instantaneously, i.e. all exposures of the fractionated course delivered at the same instant of time (Kirkby et al., 2007b). In the present model we follow more exactly the time pattern of delivery of both radiotherapy and chemotherapy schedules. Secondly, the statistical distribution of the cancer cell survival fraction in response to one fraction of radiotherapy ( $x_s$ ) is created. A particular density function was used, derived from a normalization of a modified Normal

distribution (i.e. the probability distribution is constrained within the interval 0 to 1) as follows:

$$p(x_s) = k_s x_s^n (1 - x_s)^m \exp(\alpha_s x_s). \quad (4)$$

This distribution depends on three positive, dimensionless, shape parameters ( $n$ ,  $m$  and  $\alpha_s$ ) which define the probability density function.

The quantification of the chemotherapy effect can be made by first fitting the model to radiotherapy only data, letting all the parameters of the model vary except for those parameters known *a priori*, i.e. the mean and the variance of the delay to start the treatment ( $t_{delay}$ ), extracted directly from the clinical data, and the mean of critical number of normal cells ( $N_{crit}$ ), the value being consistent with previous brain studies. Then the model is refitted to the radiotherapy plus TMZ data, keeping constant all the other parameters and allowing only the parameters ( $n$ ,  $m$  and  $\alpha_s$ ) determining the survival fraction distribution to change.

## 5.2 Scenario 2: independent cytotoxicity

We next suppose that TMZ has a direct effect on the tumour and no effect on the radiosensitivity of GBM cells. In order to develop this scenario, we add to the original model a chemotherapy module assuming that the drug kills the cancer cells independently.

As described above, the current standard chemotherapy consists of a total of 72 doses: 42 doses are administered concomitantly with radiotherapy and 30 doses are given during the adjuvant treatment. To incorporate the independent cytotoxicity of chemotherapy, Eq. (1) is modified as follows:



$$C(t_{delay}^+) = C(t_{delay}^-)x_{rt}^j x_{chemo}^i \quad (5)$$

where  $t_{delay}^+$  and  $t_{delay}^-$  represent respectively a time just after and before the treatment (days), where  $x_{chemo}$  denotes the survival fraction of cancer cells (dimensionless) after a single dose of TMZ,  $x_{rt}$  is the same as the  $x_s$  of Eqs. (3) and (4) which describes the survival fraction after each 2 Gy fraction of radiotherapy (dimensionless),  $j$  and  $i$  indicate respectively the numbers of radiation and TMZ exposures (dimensionless). It is assumed that the chemo-survival fraction ( $x_{chemo}$ ) does not depend on the dose intensity in the range of dosage used in the EORTC-NCIC study. Statistically, the chemo-survival fraction is treated in the same way as the radio-survival fraction (see Eq. (4)). The model, fitted previously to the radiotherapy only data, is then refitted to the radiotherapy plus TMZ data, where only the parameters determining the chemo-survival fraction are allowed to change.

## 6 Incorporation of the linear-quadratic model

The principal target for radiation damage is DNA and the best description, in the low-dose region (0-3 Gy) which is clinically relevant (Hall and Giaccia, 2006), is given by the LQ model:

$$x_{rt} = \exp(-\alpha d - \beta d^2) \quad (6)$$

Where  $d$  is the dose per fraction (Gy), while  $\alpha$  ( $Gy^{-1}$ ) and  $\beta$  ( $Gy^{-2}$ ) are the respective linear and quadratic coefficients for cell kill. One simplified interpretation of this equation is that the  $\alpha d$  component is due to double strand breaks, i.e. a single ionizing particle is required, while the  $\beta d^2$  component arises from single strand breaks, i.e. two ionizing particles sufficiently close in time and in distance are necessary. The dose at

which the contribution from the first and second terms are equal is given by  $\alpha/\beta$  (Gy), generally called the ‘alpha-beta ratio’. This parameter distinguishes the late-responding tissues ( $\alpha/\beta \cong 2$  Gy) in brain from the early responding tissues ( $\alpha/\beta \cong 10$  Gy). For early radiation effects, the linear component  $\alpha$  dominates at low doses, whereas for late effects, the quadratic component  $\beta$  is also relevant. GBM tumours are generally considered to behave like early responding tissues (Williams et al., 1985).

Let the total dose be  $D$  (Gy); an alternative way of representing the LQ model is given by this expression:

$$D = \frac{E/\alpha}{1 + d/(\alpha/\beta)}, \quad (7)$$

where  $E = -\ln(x_n) = \alpha D + \beta d D$ . If then  $E$  is divided through by  $\alpha$ , we obtain the definition of Biologically Effective Dose (BED):

$$BED = E/\alpha = D(1 + \frac{d}{\alpha/\beta}). \quad (8)$$

BED values are expressed in units of Gray with a subscript that denotes the numerical value of the  $\alpha/\beta$  ratio used; this is used to clarify that it is a biological dose rather than a physical dose (Fowler, 1989). Conceptually, the BED represents the physical dose required for a given effect, if the dose were to be delivered by infinitely small doses per fraction or, in the case of continuous radiation rates, at a very low dose rate (Jones et al., 2001). Note that as the dose per fraction,  $d$ , becomes very small, the number of fractions will then need to be increased to maintain the same effect, while  $E$  approximates  $\alpha D$ .

The BED has been introduced with the aim of comparing our results with other studies.

This value is particularly useful in inter- and intra-treatment comparisons and in the development of new fractionation schemes (Jones et al., 1995). It may also be used to assess the relative contribution of chemotherapy (Jones and Dale, 2005).

In order to give a radio-biological connotation to the cell survival fraction, a fundamental parameter of the main model, we replace its analytical expression  $x_s$  with Eq. (6). To allow the generation of a population of patients via Monte Carlo simulation we assume that  $\alpha$  and  $\beta$  are normally distributed. This assumption is consistent with previous studies where these parameters are usually assumed to follow a normal distribution (Jones and Dale, 1999). Normally distributed values of  $\alpha$  and  $\beta$  can lead to a skewed distribution in  $x_{rt}$ , consistent with published data (Björk-Eriksson et al., 1998). It should be noted that using a normal distribution we may occasionally generate implausible values of  $x_{rt}$ . Hence we ignore those values greater than one. The number of rejected values depends on the number of values generated and the mean and variance of  $\alpha$  and  $\beta$ . In the following work, the values of  $\alpha$  and  $\beta$  used very rarely if ever gave rise to  $x_{rt}$  greater than one.

## **7 Other changes to the original model**

### **7.1 Tumour doubling time distribution**

As noted above, this model has several adjustable parameters which vary according to specific probability density functions. Two of these variables involve time, namely, the time delay to commence treatment and the tumour doubling time. In the original model these parameters were assumed to be normally distributed. It is more appropriate to use different probability density functions, since realistic time values lie in the interval  $[0, +\infty]$ . Thus, in the present model the doubling time can be also described either by a

Gamma distribution, a Weibull distribution, or a Log-Normal distribution.

Computationally, normal variates are generated using the Box-Muller method, which requires two uniform random variates, whereas these distributions can be simply generated through inversion of the relative cumulative density function.

## 7.2 Definition of the tumour regrowth time

The present model can also predict the regrowth time of the tumour. The tumour regrowth time is defined as the time taken to re-establish the same number of cancer cells as just before the commencement of the treatment. Eqs. (1) and (3) determine the regrowth process, and it is also assumed that the rate constant for cancer cell growth ( $k_c$ ) is the same as that applied before the treatment.

When  $C(t_{surv}) = C(t_{delay}^-)$ , the tumour regrowth time is

$$t_{regrowth} = t_{surv} - t_{delay}, \quad (9)$$

where  $t_{delay}$  is the time delay before the treatment and  $t_{surv}$  is the overall survival (days).

The tumour regrowth time is then estimated for each single patient and the relative distribution among the population of patients is approximated using a Gaussian kernel density function. For further statistical analysis see Kirkby et al. (2007b).

## 8 Results

### 8.1 Model fit to clinical data

As in our previous publications, the model is capable of successfully fitting the clinical data of the radiotherapy only arm (Fig. 2). The population parameter values, expressed as mean and variance, are given in Table 1.

For the assumption of pure radiosensitization (Scenario 1), the model fitting achieved in the radiotherapy plus TMZ arm is excellent as shown in Fig. 3. The resulting parameter values are summarized in Table 1. The closeness of the fit is equally good along the entire survival curve. This indicates a close agreement between the model and the real data assuming that TMZ increases tumour cells radiosensitivity.

For the assumption of independent cytotoxicity (Scenario 2), Fig. 4 shows the model fit. The survival curve resulting from fitting in Scenario 2 has an important divergence compared with the survival curve achieved in Scenario 1. Clearly observable, by visual inspection of the survival curves given in Fig. 4, is the presence of a substantial overestimation of the model at the level of median survival when it is assumed that TMZ anti-tumour activity is due only to its cytotoxicity (as represented in Eq. (5)). It would be expected that by adding more parameters in the model, the fit would be superior in Scenario 2 since the model with the higher number of parameters generally provides a better objective function. According to the Akaike (AIC) and Schwarz (SC) parsimony criteria, and assuming that the model error is normally and independently distributed, Scenario 1 is superior: in Scenario 1  $AIC = 6.21$  and  $SC = 6.56$  versus  $AIC = 6.36$  and  $SC = 6.71$  for Scenario 2 (Akaike, 1974; Schwarz, 1978).

## 8.2 Distribution of the survival fraction

In Scenario 1, the comparison of the survival fraction distribution after a single fraction of radiotherapy shows a change in the shape attributable to the radiosensitizing effect of TMZ (Fig. 5). The distribution of the pure radiation effect has a mean survival fraction of 0.77 and a standard deviation of 0.11, while the distribution of the combined treatment has a mean value of 0.68 and a standard deviation of 0.14. The addition of

TMZ causes a reduction of 12 % in the mean value and an increase of 27 % in the standard deviation of the probability density function of the survival fraction. The most clinically relevant difference is on the left tail of the distribution, where the tail starts to flatten later. This indicates that the effect associated with one 2 Gy fraction of radiation is enhanced by TMZ radiosensitization, so that more patients have tumours sensitive enough to be cured. The efficiency of TMZ in improving radiotherapy response depends on the number of sensitized fractions, and consequently it may be schedule-dependent. The probability density function for the chemo survival fraction, assuming that TMZ directly kills tumour cells, is presented in Fig. 6. The TMZ survival fraction distribution is extremely skewed, with a mean of 0.909 and a standard deviation of 0.083. These observations imply that a significant proportion of the population does not derive direct effect from TMZ, and that a dose of TMZ is not necessarily sufficient to cause significant cell kill.

### 8.3 Radiobiological parameter values

The addition of the LQ model does not alter the quality of the fit. The fit achieved in Scenario 1 is substantially better than in Scenario 2 in line with the above presented findings. In addition, the LQ equation allows us to estimate the *in vivo*  $\alpha/\beta$  ratio for high-grade brain tumour cells, values that are very difficult to obtain experimentally. The reasons for this variability are not completely known. Nevertheless it is known that several factors are involved, for example low tumour oxygenation, fast regrowth kinetics and an efficient DNA repair system are indices of a high *in vivo* tumour radioresistance (Perez and Brady, 1998; Steel, 2002). In addition, the cell response to radiation considerably varies through the cell cycle (Steel, 2002). The model fitting to

the radiotherapy only arm produces a respective mean value of  $0.102 \text{ Gy}^{-1}$  for  $\alpha$  and of  $0.008 \text{ Gy}^{-2}$  for  $\beta$ , that result in an  $\alpha/\beta$  ratio of 12.5 Gy. This value is in line with other published data for rapidly growing and radioresistant tumours, e.g. 10-30 Gy (Steel, 2002).

When we fit the model to the radiotherapy plus TMZ arm the  $\alpha/\beta$  ratio value decreases to 3.1 Gy, with a respective mean value for  $\alpha$  of  $0.094 \text{ Gy}^{-1}$  and for  $\beta$  of  $0.03 \text{ Gy}^{-2}$ . As the addition of TMZ influences the survival fraction distribution enhancing the radiation response, we can reasonably expect a reduction of the  $\alpha/\beta$  ratio. Variations in the  $\alpha/\beta$  ratio cause large variations in the shape of the survival curves, which are at the origin of the differential effect connected with radiosensitivity.

It is clearly not meaningful to compare BED using different  $\alpha/\beta$  ratios. In fact it is never possible to match two different regimens to be equivalent for both early- and late-responding tissues. However it can give a measure of the potential biological dose delivered to the tumour for each single arm of the trial. Calculating the BED using these  $\alpha/\beta$  ratios, for a physical dose of 60 Gy delivered in 30 fractions, gives a BED for radiotherapy only of  $69.90 \text{ Gy}_{12.5}$ , while for radiotherapy plus TMZ it is  $98.71 \text{ Gy}_{3.1}$ .

#### 8.4 Temozolomide effect on radiation sensitivity

The synergistic action of TMZ clearly causes a decrease (by 75.2 %) in the  $\alpha/\beta$  ratio, from values of early responding tissues to values of late responding tissues. This produces a different shape of the cell survival dose-response curve as shown in Fig. 7. The survival curve for radiotherapy only is almost a straight line with slope  $\alpha$ , whereas the survival curve for radiotherapy plus TMZ tends to be more curved.

Other points are visible from Figs. 8 and 9: TMZ influences slightly the linear component distribution, whereas it affects substantially the quadratic component distribution (mean increase by a factor of 2.75). These results can be read according to the LQ model interpretation in which cell death is due both to directly lethal events and to the accumulation of sublethal lesions, leading to lethal chromosome breaks.

High-grade brain tumour cells are extremely radioresistant, and radiation lethality is due essentially to direct lethal lesions. TMZ seems to render GBM cells more sensitive to radiation exposure. The implication of this is that with TMZ either the sublethal damage contributes significantly to cell killing or some potentially repairable damage sites are ‘fixed’ by TMZ before repair can occur. Furthermore a lower  $\alpha/\beta$  ratio is usually associated with a slow proliferation rate (Hall and Giaccia, 2006). This suggests that TMZ may also affect the regrowth kinetics and inhibit the DNA synthesis, i.e. reduces repopulation during radiotherapy.

The apparent change in the  $\alpha/\beta$  ratio suggests that the concomitant use of TMZ may be further enhanced by adopting new dose escalation or dose fractionation strategies, providing tolerance of the normal brain is not exceeded.

### **8.5 Tumour regrowth time**

The model predicts the regrowth time distribution of tumours for both arms of the EORTC-NCIC study. The tumour regrowth time allows us to infer the natural history of the tumour and quantify the effects of TMZ addition.

Our estimates of the tumour regrowth time distribution are shown in Fig. 10. The mean tumour regrowth time for the radiotherapy only arm is 281.4 days and median 218.3



days. With radiotherapy plus TMZ, assuming synergy, the tumour regrowth time is substantially higher: mean and median respectively of 436.8 days and 345.4 days. Therefore the distribution produced by the addition of TMZ is more spread (standard deviation 369.7 days) over the population of patients than with radiotherapy only (standard deviation 283.2 days). Note that in this analysis we generated 2000 patients so that the approximated distribution shape was clearly visible and not influenced by those sporadic patients far from the population mean value.

## 9 Discussion

The model discussed here is an attempt at representing *in vivo* effects of TMZ combined with radiation. Using the model, we have investigated two possible approaches to determine the contribution of TMZ to radical radiotherapy. We first focused on TMZ-mediated radiosensitization. Then we have sought to develop an independent module to analyse another potential process: independent TMZ cytotoxicity. The parameter values were determined by fitting the model to both arms of the EORTC-NCIC study.

The model suggests that TMZ enhances the therapeutic efficacy of radiation by radiosensitising GBM cells. All of the survival advantage from radiotherapy plus TMZ can be explained by this mechanism. Therefore, the concurrent administration of this agent seems to have greater anti-tumour effect compared with sequential administration.

In other tumours (e.g. cervical cancer), low dose chemotherapy concurrently with radiotherapy has clearly been demonstrated to produce a radiosensitizing effect (Dubay et al., 2004; Keys et al., 1999; Rose et al., 1999). Furthermore, a recent retrospective clinical study of patients with GBM treated only with concurrent radiotherapy and TMZ, and omitting the adjuvant TMZ component, suggests that the concomitant rather

than the adjuvant phase is the most efficacious part, raising the question of the optimal timing of chemotherapy in the treatment of GBM (Sridhar et al., 2009).

The model is also capable of extracting radiobiological information from the clinical patient data. In particular, using the model we have estimated the average value of the *in vivo*  $\alpha/\beta$  ratio for a population of glioma cells, and it appears to be biologically consistent. We have also established the effects of TMZ on the radiosensitivity of the tumour. The BED concept can then be used to evaluate different dose escalation schemes of radiotherapy and to develop new clinical trial strategies.

### 9.1 TMZ and GBM cells radiosensitivity

The probability distribution of the survival fraction, after fitting the model to the radiotherapy alone data of the EORTC-NCIC study, is consistent with our previous results. In Kirkby et al. (2007b) we derived a mean value of the survival fraction of 0.80 from the Medical Research Council BR02 trial. In this study, our estimate of 0.77 probably reflects the influence of different patient characteristics between the two trials. The similarity of the modelling parameters between the EORTC-NCIC study and our previously published radiotherapy alone fitting is not surprising since the Kaplan-Meier survival curves are also similar.

With respect to the TMZ and radiotherapy arm of the EORTC-NCIC study, the fit achieved in Scenario 1 is substantially better than the one that could be achieved with Scenario 2, which suggests a TMZ-mediated radiosensitization. Therefore the adjuvant schedule may have only a marginal benefit compared to the concomitant phase. This result is consistent with published data of *in vitro* and *in vivo* radiosensitization of TMZ (Chakravarti et al., 2006; Kil et al., 2008). The model suggests that TMZ increases

radiosensitivity with a dose enhancement factor in surviving fraction of 0.11, in line with the result reported by Kil et al. (2008) of 0.10 for human glioma and breast tumour cells. More specifically, the survival fraction distribution after one dose of radiation is less skewed with the addition of TMZ (Fig. 5). This indicates that TMZ increases the proportion of patients with radiosensitive tumours.

There are several possible mechanisms through which TMZ can enhance radiosensitivity. For example, TMZ may redistribute the cells into a radiosensitive phase of the cell cycle. In general, cells exhibit the greatest radiosensitivity in the M and G2 phases, and resistance in the S phase (Hall and Giaccia, 2006; Steel, 2002). Another possible source is the abrogation of the G2 checkpoint. TMZ-mediated radiosensitization may also involve an increased degree of radiation-induced apoptosis (Chakravarti et al., 2006). In addition, TMZ may enhance the radiotherapy efficacy by increasing the number of double-strand DNA breaks after radiation exposure. Alternatively, the concomitant administration of TMZ may inhibit the repair of double-strand DNA breaks (Chakravarti et al., 2006; Kil et al., 2008).

The model can be used to study in depth the mechanisms behind TMZ-mediated radiosensitization. Our *in vivo* estimates of the  $\alpha/\beta$  ratio for both arms of the EORTC-NCIC study are consistent with the average values of the  $\alpha/\beta$  ratio estimated for glioma cell lines (Qi et al., 2006; Steel and Wheldon, 1991; Steel, 2002).

The probability distributions of the radiobiological parameters have a Gaussian shape consistent with published data of *in vitro* radiosensitivity. It might be expected that the  $\alpha$  component, which describes the double-strand DNA breaks, would be more affected by TMZ radiosensitization. The model suggests a lower value of the  $\alpha/\beta$  ratio when TMZ

is administered concomitantly with radiotherapy; in particular it predicts a great change in the mean value of  $\beta$ . This observation can be explained by the fact that TMZ may cause single-strand breaks in close proximity to radiation-induced single-strand breaks on adjacent strands of DNA (Chakravarti et al., 2006). If these TMZ-induced single-strand DNA breaks are sufficiently close to the radiation-induced ones, it could be then possible that they convert into double-strand breaks.

In a separate modelling study, Jones and Sanghera (2007) have determined the radiobiological parameters for high-grade glioma based on the 45 and 60 Gy arms of the BR02 trial and our previously published estimate of the median surviving fraction after 2 Gy of 0.83 (Burnet et al., 2006). Jones and Sanghera (2007) estimated an  $\alpha/\beta$  ratio of 9.32 Gy and a median  $\alpha$  value of  $0.077 \text{ Gy}^{-1}$  and  $\beta$  of  $0.008 \text{ Gy}^{-2}$ . The slightly higher  $\alpha$  value and consequently  $\alpha/\beta$  ratio in the present study probably reflect that Jones and Sanghera's work is based on tumour regrowth time and the present work actually predicts patient survival time directly. Interestingly, the  $\beta$  values are the same. In their paper, the equivalent BED for TMZ was estimated to be  $11.03 \text{ Gy}_{9.3}$  (equivalent to a radiation dose of 9.1 Gy given in 2 Gy fractions). This value depends on their estimated average  $\alpha/\beta$  ratio and therefore it is not directly comparable with our results.

## 9.2 Tumour regrowth time distribution

Our modelling suggests that the addition of TMZ produces a considerable extension of the tumour regrowth time. The model can be used to compare tumour regrowth time and treatment duration. Our evaluation suggests that the increase in the tumour regrowth time does not match the duration of either the concomitant (i.e. 42 days) or the adjuvant schedule (i.e. 168 days). The growth delay after the combined treatment is more than

the sum of the relative growth delays caused by individual treatments, calculated as a fraction of the radiotherapy only treatment, used as a normalization control factor. This may indicate the presence of a synergistic effect between radiation and TMZ which results in a greater than additive response. This finding is in line with the data reported by Kil et al. (2008) concerning *in vivo* tumour growth delay in mice bearing xenografts treated with radiotherapy and TMZ.

Another aspect visible from the tumour regrowth time distribution is that the number of patients that have not benefited from radiotherapy (i.e. zero regrowth time) in the radiotherapy only arm is decreased by 60 %. This means that TMZ may affect even those tumours which are considered to be highly resistant to radiation.

### 9.3 Optimising radiotherapy and chemotherapy combination

Radiotherapy with concomitant and adjuvant TMZ has become normal practice for radical treatment (i.e. with curative intent) in patients with GBM. However, this schedule achieves a cure in only a small proportion of patient treated and further therapeutic and clinical improvements are required. It is also not clear whether this regimen is optimal in terms of the maximum benefit from the combined treatment.

The incorporation into the model of chemotherapy, real fractionation patterns and radiobiology has allowed us to investigate the role of TMZ, in particular the relative contributions of the concurrent compared with the adjuvant phase. It should be noted that the EORTC-NCIC study was not designed to explore this issue directly. Moreover, most of the current and future clinical trials are addressed to examine treatment intensification of TMZ and to increase the number of maintenance cycles of TMZ (Clarke et al., 2009; Franceschi et al., 2007; Tosoni et al., 2007; Villano et al., 2009).

Patient opinion is also important in developing clinical trials, and in general patients with GBM are reluctant to reduce TMZ use. Although a study is in progress including a trial arm where adjuvant TMZ is not given, i.e. comparing radiotherapy with concurrent plus adjuvant TMZ versus radiotherapy with concurrent TMZ alone, this is in patients with a different, less aggressive type of glioma (anaplastic astrocytoma) (Stupp et al., 2009). The results will not necessarily be transferable to patients with GBM.

Our results suggest that particular focus on the use of TMZ as a radiosensitizer would be worthwhile. Although more clinical studies are required to address these questions, our modelling can partially avoid the need to resort to large-scale clinical trials that might be both difficult and time-consuming.

#### **9.4 Other characteristics of the model**

In Scenario 2 the divergence between the model prediction and the real clinical outcome suggests that TMZ does not cause a significant independent cell kill. This result is dependent on the validity of Eq.(5) and the assumptions required within the model.

Despite the excellent results obtained from the administration of TMZ, not all patients with GBM benefit from TMZ. Previous pre-clinical and clinical studies have pointed out at a strong relationship between the efficacy of alkylating chemotherapy and functional inactivation of the O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) repair enzyme, also known as O<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT). MGMT is a DNA repair protein which is capable of removing alkyl groups from the O<sup>6</sup> position of guanine. Silencing of the MGMT promoter results in a low expression of this gene. Hegi et al. (2005) reported that silencing of the MGMT promoter by methylation was an independent predictor of benefit from TMZ in a subgroup of patients enrolled in the

EORTC-NCIC study (Gorlia et al., 2008).

At present, the model does not explicitly include this prognostic factor. Because of the excellent fit to the real clinical data reached in Scenario 1, we have made no attempt to link the chemo-survival fraction to the MGMT promoter status. However, this term can be easily integrated in the model as a multiplicative factor of  $x_{chemo}$  in Eq. (5). For 55 % of the patient population this factor is expected to be equal to one and for the remaining 45 % less than or equal to one, where these percentages are in accordance with the results reported by Hegi et al. (2005).

In the model, we have also deliberately assumed that the survival fraction after one dose of TMZ is not dependent on the dose or on the dose density. This assumption is related to the purpose of the model and would need to change if we subsequently want to test different dose-time chemotherapy schedules. However, we feel justified because the main dose difference is between the concomitant phase (i.e. 75 mg/m<sup>2</sup>) and the adjuvant cycles (i.e. 150-200 mg/m<sup>2</sup>), and in the trial only 47 % of patients completed all the six planned cycles of adjuvant TMZ. If anything, this assumption is likely to overestimate the cytotoxic effect, since two thirds of the doses are given at the lower dose.

The model has also been extended to include the basic radiobiology via the linear quadratic equation. We have not attempted to include in the LQ model other terms, e.g. cells repopulation, hypoxia and normal tissue responses. This level of complexity is not required for a population-based model like the one presented in this work, which is mainly designed to predict survival. However, these processes may alter some parameter values. For example, the cells newly produced during treatment specifically affect the tumour doubling time, and consequently the model prediction of the tumour

regrowth time. The oxygen level influences the radiation cell kill; hypoxic tumours are known to be radioresistant. In the future, it is intended to develop a separate DNA damage-repair model, which includes the oxygen concentration as a factor that fixes DNA strand breaks induced by radiation.

## 10 Conclusion

The model presented in this paper is a development of the model previously designed to describe the effects of radiotherapy in patients with high-grade brain tumours.

Chemotherapy has been included either as a direct factor that can affect radiation response or as an independent source that can kill tumour cells. We also integrated the linear quadratic equation to analyse the *in vivo* effects of both radiotherapy and chemotherapy.

The comparison of the *in silico* survival curve with real data from the EORTC-NCIC study demonstrates that the model can qualitatively represent the clinical reality. In particular, the incorporation into the model of chemotherapy has raised some important questions regarding the mechanism of action of TMZ and has highlighted the need for some clinical reconsiderations. Some of these questions would be extremely difficult and expensive to answer by other means.

The results suggested that TMZ enhances the therapeutic efficacy of radiation in GBM cells mostly when administered concurrently. Therefore, the activity of adjuvant TMZ as a single-agent seems to have a more marginal therapeutic benefit compared with the concurrent phase. More clinical studies and further model developments are required to validate this prediction.



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## Nomenclature

### *Latin letters*

BED	Biologically Effective Dose	(Gy <sub><math>\alpha/\beta</math></sub> )
$C(t)$	number of cancer cells in the brain	(cells)
$C_0$	number of cancer cells in the brain at presentation	(cells)
$d$	dose per fraction of radiotherapy	(Gy)
$D$	total dose of radiotherapy	(Gy)
$E$	radiation effect	(dimensionless)
$i$	number of doses of chemotherapy	(dimensionless)
$j$	number of fractions of radiotherapy	(dimensionless)
$k_c$	rate constant for cancer cell growth	(days <sup>-1</sup> )
$k_n$	rate constant for normal cell damage	((cells · days) <sup>-1</sup> )
$k_s$	normalization constant of the survival fraction distribution	(dimensionless)
$m$	shape parameter of the survival fraction distribution	(dimensionless)
$n$	shape parameter of the survival fraction distribution	(dimensionless)
$N(t)$	number of normal cells in the brain	(cells)
$N_{crit}$	critical number of normal cells required for patient to remain alive	(cells)

$N_0$	number of normal cells left at presentation	(cells)
$t$	Time	(days)
$t_D$	tumour doubling time	(days)
$t_{delay}$	delay to start the treatment	(days)
$t_{regrowth}$	tumour regrowth time	(days)
$t_{surv}$	overall survival time from presentation	(days)
$x_{chemo}$	cancer cell survival fraction in response to one fraction of chemotherapy	(dimensionless)
$x_s - x_{rt}$	cancer cell survival fraction in response to one fraction of radiotherapy	(dimensionless)
<i>Greek letters</i>		
$\alpha$	linear component of the linear-quadratic model	(Gy <sup>-1</sup> )
$\beta$	quadratic component of the linear-quadratic model	(Gy <sup>-2</sup> )
$\alpha/\beta$	Dose at which cell killing by the linear and quadratic components are equal	(Gy)

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# Tables.

**Table 1.** Details of values of the variables from the fit to the EORTC-NCIC trial 26981-22981/ CE.3. Note that between the two arms, radiotherapy alone and radiotherapy plus TMZ, only the parameters determining the survival fraction ( $n$ ,  $m$  and  $\alpha_s$ ) are allowed to change. For details of the other parameter values see text.

	Radiotherapy alone		Radiotherapy + TMZ (Scenario 1)	
	$\mu$	$\sigma^2$	$\mu$	$\sigma^2$
$n$ (-)	9.08	-	4.28	-
$m$ (-)	2.2	-	2.34	-
$\alpha_s$ (-)	1.13	-	3.19	-
$N_0$ (cells)	$1.48 \times 10^{12}$	$5.27 \times 10^{19}$	"	"
$N_{crit}$ (cells)	$1.0 \times 10^{12}$	$8.27 \times 10^{22}$	"	"
$k_n$ ((days · cells) <sup>-1</sup> )	$3.35 \times 10^{-12}$	$2.34 \times 10^{-26}$	"	"
$t_D$ (days)	23.88	186.2	"	"
$t$ ( <sup>a</sup> ) <sub>delay</sub> (days)	52.15	28	43.4	11

<sup>a</sup> Parameters from clinical series.

### Captions.

**Fig. 1.** Kaplan-Meier survival plots according to the treatment group for half data set of the EORTC-NCIC trial 26981-22981/CE.3.

**Fig. 2.** Kaplan-Meier survival plot for 143 patients treated with radiotherapy only from the EORTC-NCIC trial 26981-22981/CE.3, and the fitted model.

**Fig. 3.** Kaplan-Meier survival plot for 144 patients treated with radiotherapy plus TMZ from the EORTC-NCIC trial 26981-22981/CE.3, and the fitted model assuming pure radiosensitization by TMZ. Note that only the parameters determining the survival fraction after one dose of radiotherapy are allowed to change.

**Fig. 4.** Kaplan-Meier survival plot for 144 patients treated with radiotherapy plus TMZ from the EORTC-NCIC trial 26981-22981/CE.3, and the fitted model using the chemotherapy module. This assumes that the effect of TMZ is entirely due to direct cytotoxicity which is independent of radiotherapy.

**Fig. 5.** Comparison of the probability distributions of survival fraction, after a single 2 Gy fraction of radiotherapy, after fitting the model separately to the radiotherapy only data and the radiotherapy plus TMZ data. The graph shows the difference in the shape which is produced by the synergistic effect of TMZ.

**Fig. 6.** Probability distribution of the modelled chemo-sensitivity resulting from fitting to the radiotherapy plus TMZ data.

**Fig. 7.** Equivalent of *in vitro* dose-response curves for radiotherapy only and radiotherapy plus TMZ. Note that the addition of TMZ produces a lower  $\alpha/\beta$  ratio. For this type of experiment, or its equivalent, the radiotherapy is given as a single fraction of variable dose.

**Fig. 8.** Distributions of the linear component  $\alpha$ , resulting from fitting to radiotherapy only data and radiotherapy plus TMZ data. Note that values greater than one are ignored.

**Fig. 9.** Distribution of the quadratic component  $\beta$ , resulting from fitting to radiotherapy only data and radiotherapy plus TMZ data. Note that values greater than one are ignored.

**Fig. 10.** Probability distributions, approximated with a Gaussian kernel function using 100 equally spaced points, of the tumour regrowth time, resulting from the fitting to radiotherapy only data and radiotherapy plus TMZ data assuming a synergistic effect between radiotherapy and TMZ. For radiotherapy alone the mean is 281.4 days and the median is 218.3 days. For radiotherapy plus TMZ the mean is 436.8 days and the median is 345.4 days. Note that the model population size is of 2000 patients.

Fig 1

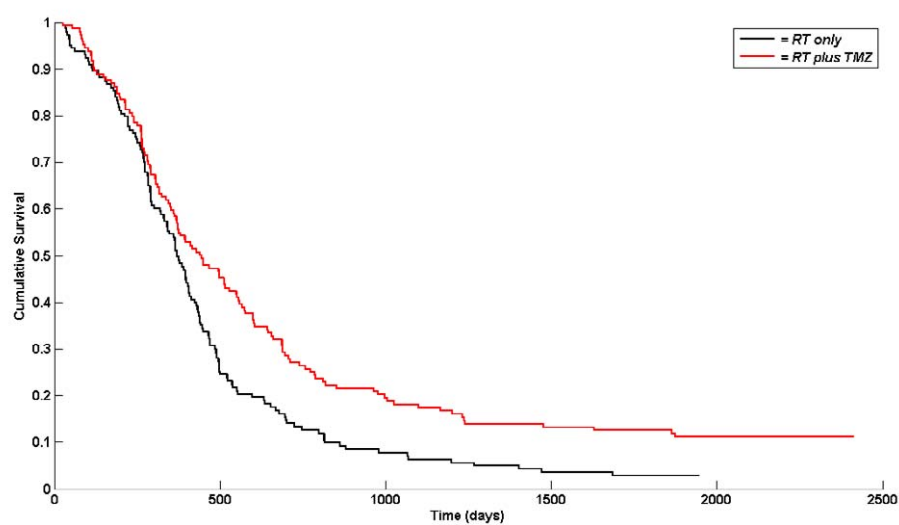


Fig 2

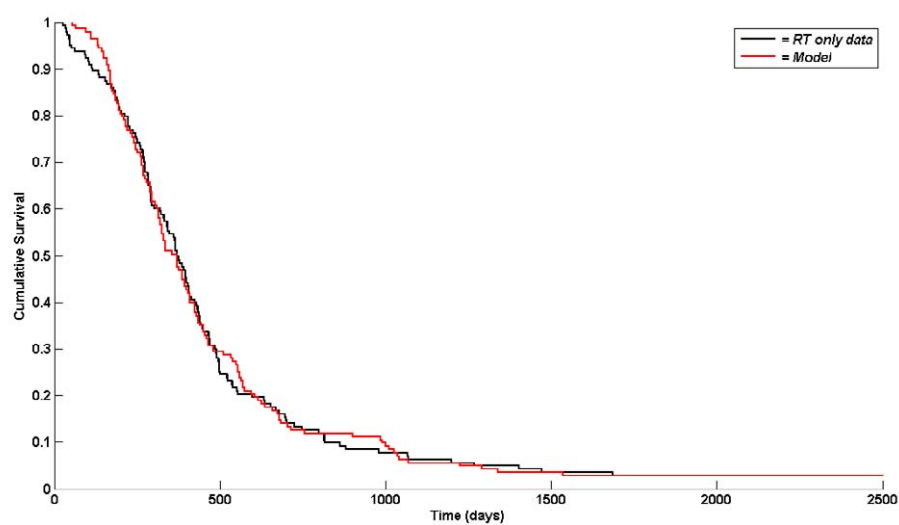


Fig 3

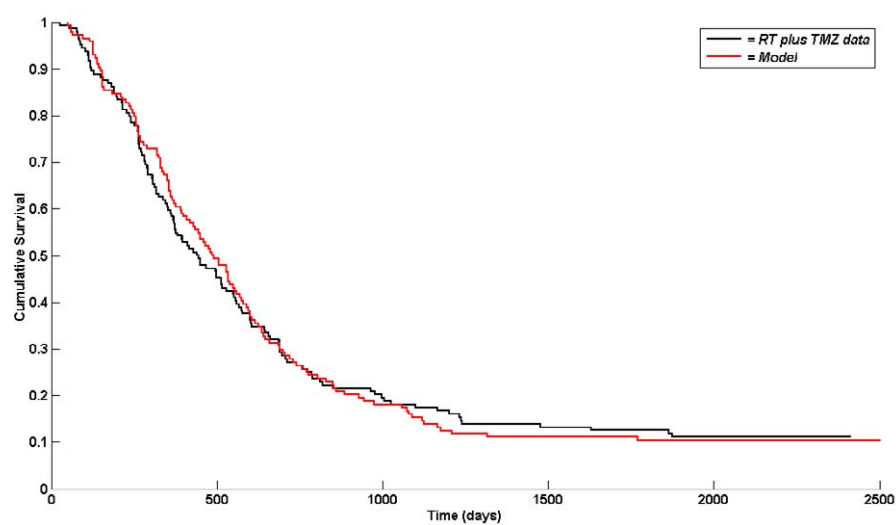




Fig 4

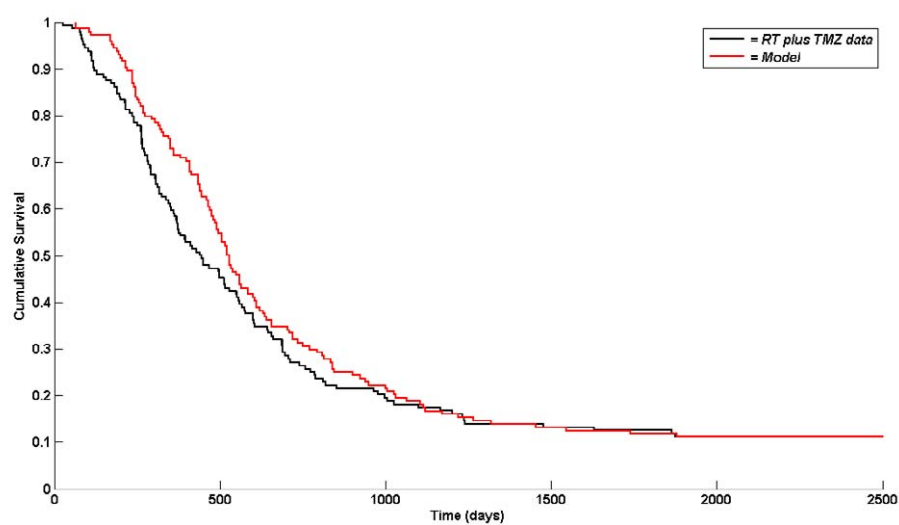


Fig 5

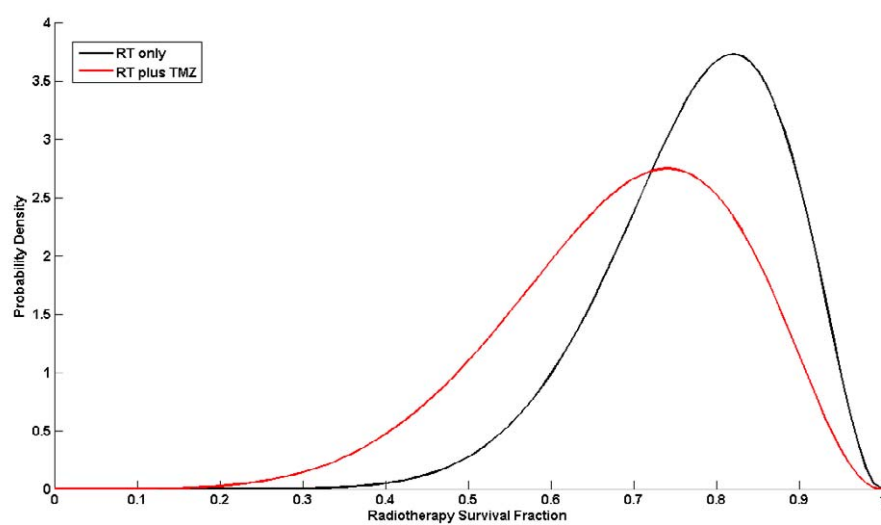


Fig 6

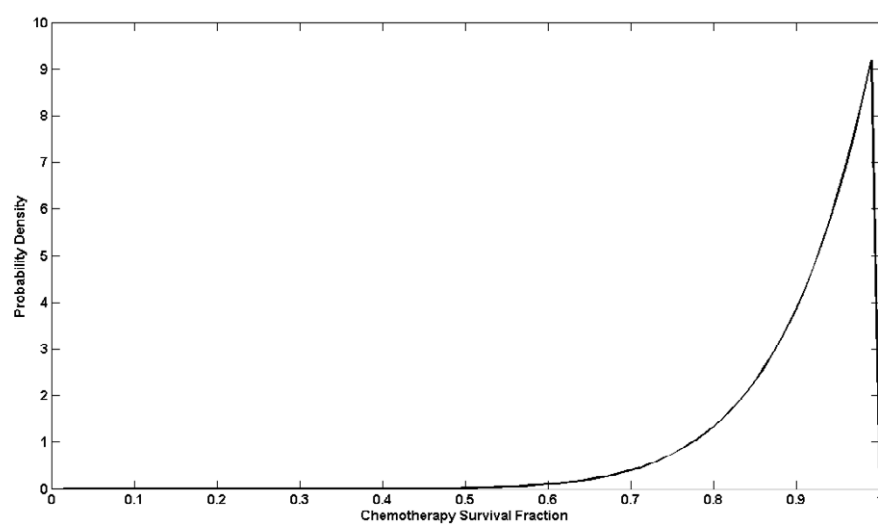


Fig 7

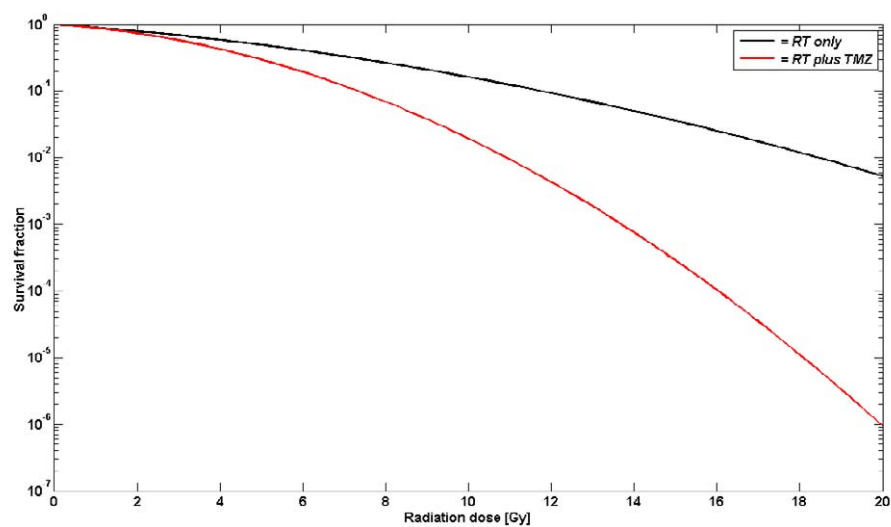


Fig 8

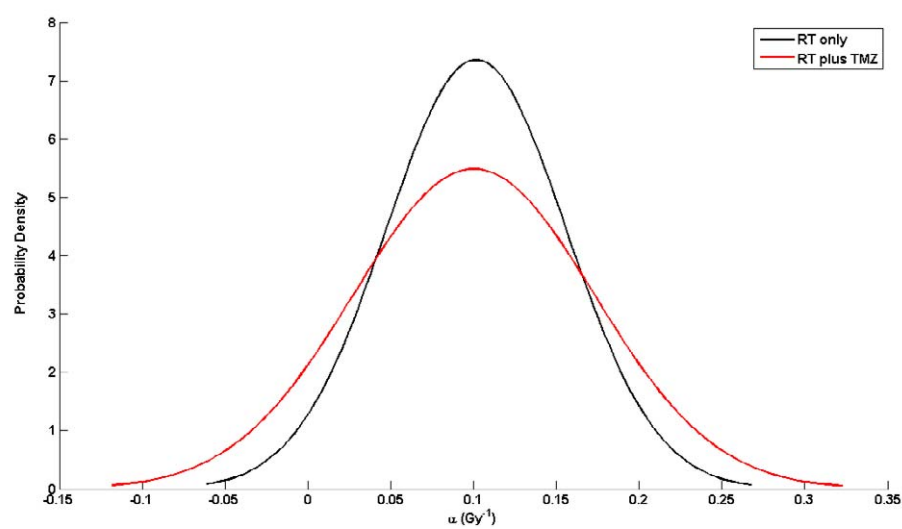


Fig 9

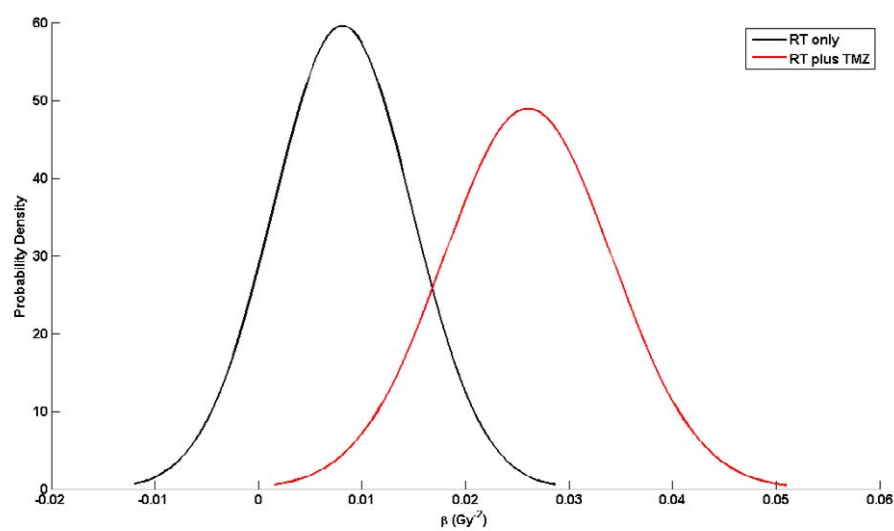


Fig 10

