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**Poly(ADP-ribose) polymerase-1 (PARP-1) pharmacogenetics,
activity and expression analysis in cancer patients and healthy
volunteers**

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Short Title : PARP expression and activity

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Synopsis

There is a wide inter-individual variation in PARP activity, which may have implications for health. We investigated if the variation (i) is due to polymorphisms in the *PARP-1* gene or PARP-1 protein expression and (ii) affects patients' response to anticancer treatment.

We studied 56 healthy volunteers (HV) and 118 cancer patients (CP), with supporting *in vivo* experiments.

PARP activity ranged between 10–2600 pmol PAR/10⁶ cells and expression between 0.02–1.55 ng PARP-1/μg protein. PARP-1 expression correlated with activity in HV ($R^2=0.19$, $P=0.003$) and CP ($R^2=0.06$, $P=0.01$). A short CA repeat in the promoter was significantly associated with increased cancer risk (OR, 5.22; 95% CI, 1.79–15.24). PARP activity was higher in men than women ($P=0.04$) in the HV. Male mice also had higher PARP activity than females or castrated males. Estrogen supplementation activated PARP in PBMCs from female mice ($P=0.003$) but inhibited PARP in their livers by 80%.

PARP activity and expression were not dependent on the investigated polymorphisms but there was a modest correlation of PARP activity with expression. Studies in the HV revealed sex differences in PARP activity, confirmed in mice and associated with sex hormones. Toxic response to treatment was not associated with PARP activity and/or expression.

Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is involved in DNA repair, genomic stability, transcription control, cell death and proliferation (reviewed in [1, 2]). Binding of PARP-1 at DNA breaks activates the enzyme to cleave NAD⁺ and create long homopolymers of ADP-ribose attached to both PARP-1 itself and histone tails at the vicinity of the break, thereby “flagging” the damage to the repair machinery. PARP-1 knockout mice, and the cells derived from them, are hyper-sensitive to DNA methylating agents, topoisomerase I poisons and ionising radiation. These agents are used in the treatment of cancer and PARP inhibitors increase their anticancer activity (reviewed in [1, 2]). Paradoxically, PARP activity can also promote cell death in non-replicating normal cells that are not so dependent on rapid DNA repair. In such cells and tissues a burst in reactive oxygen species formation following ischemia-reperfusion injury, infection and inflammation leads to DNA breaks that activate PARP-1 resulting in rapid and catastrophic NAD⁺ and ATP depletion and subsequent cell death (reviewed in [3]). Clearly, PARP activity has implications in human health and disease and response to anticancer therapy. Large inter-individual differences in PARP activity in peripheral blood mononuclear cells (PBMCs) have been reported in both healthy volunteers and cancer patients [4-7]. High PARP activity may promote DNA repair and genomic stability in normal cells as well as cancer cells thus can lead to resistance to DNA-damaging anticancer treatment. However, low PARP activity may lead to reduced pro-inflammatory mediators, tissue damage, necrosis and reperfusion injury.

Little is known about the potential underlying mechanisms responsible for the variation in PARP-1 activity and expression. There are at least 60 reported single nucleotide polymorphisms (SNPs) in the *PARP-1* sequence (<http://snp500cancer.nci.nih.gov>). One of these polymorphisms in the promoter region is a microsatellite polymorphic DNA fragment, consisting of a variable number of CA repeats [8] that may facilitate transcription from the promoter *via* the formation of DNA quadruplex structures [9]. Furthermore, the CA microsatellite is located close to the binding site of the transcription factor Yin Yang 1 (YY1), and this may also contribute to the regulation of transcription [10, 11]. The common T2444C SNP (at a frequency of 5-33%), resulting in an amino acid substitution, Val762Ala, in the PARP-1 catalytic domain, has been reported to reduce PARP-1 catalytic activity by 30-40% and to be associated with various cancers [4, 12-15].

Numerous studies suggest a correlation between PARP activity and age. A positive correlation between specific PARP activity and mean maximal life span in 13 mammalian species as well as a decrease in PARP activity with age in humans and rats was previously reported [16]. In contrast, enhancement of PARP activity was reported in brains of old adult animals compared with young controls [17] and in lymphoblastoid cell lines derived from centenarians [7].

Patients vary in their toxic and therapeutic response to treatment due to several different factors and pharmacogenetics may be used to predict toxicity and response allowing more tailored drug treatment. Recent clinical trials with PARP inhibitors indicate that suppression of PARP-1 activity can have a profound effect on chemotherapy induced toxicity [18] as well as the efficacy of chemotherapy [19]. An understanding of the genetic determinants of PARP-1 activity and its relation to patients' response was investigated in this study.

Our aim was to further evaluate the inter-individual differences in PARP activity and determine the underlying mechanisms responsible for the variation in terms of PARP-1 protein expression, polymorphisms in the *PARP-1* gene and demographic factors such as age and sex. We investigated the underlying mechanisms by measuring PARP-1 polymorphisms, expression and activity in PBMCs from 118 cancer patients and 56 healthy volunteers, with supporting *in vivo* studies. We also studied if PARP-1 activity contributes to patients' response to treatment in terms of toxicity and if particular types of malignancy are associated with higher or lower PARP activity.

Materials and Methods

Chemicals

β -estradiol 17-valerate and all routine chemicals and tissue culture reagents were supplied by Sigma (Dorset, UK) unless stated otherwise. AG014699 was a kind gift from Dr Zdenek Hostomsky (Pfizer Oncology, La Jolla, USA).

Cell line

Chronic myelogenous leukaemia K-562 cells obtained from ATCC (CCL-243, Manassas VA, USA) were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% antibiotic-antimycotic at 37°C in an atmosphere of 5% CO₂ in air. Cells were confirmed *Mycoplasma* negative by regular testing (Mycoalert; Cambrex, Charles City, IA, USA).

Hormonal manipulation in mice

The animal study was conducted in accordance with national law and institutional guidelines under a protocol approved by local Ethics Committee. CD-1 mice, 8-10 weeks of age (Charles River Laboratories, Wilmington, MA, USA), were treated as follows: male untreated controls (n = 9), castrated untreated males (n = 9), castrated males treated with 4 mg β -estradiol 17-valerate per mouse dissolved in corn oil (n = 15) by a single intramuscular (IM) injection on the day of castration, untreated females (n = 9) and females (n = 15) treated with β -estradiol 17-valerate as above. Animals were killed 6 days later and blood from controls (n = 3) and treated animals (n = 5) was pooled prior to collection of PBMCs. Livers from control female mice (n = 3) and from estradiol treated female mice (n = 3) were also collected for analysis and stored in -80°C.

Human subjects

The research protocol for the PARP clinical study was approved by the local Ethics Committee. The study included cancer patients (CP) newly diagnosed with solid tumours who were referred to Northern Centre for Cancer Treatment (NCCT) between February 2007 – December 2008 and healthy volunteers (HV). Subjects supplied a blood sample (10-20 ml) and data including date of birth, sex, ethnicity, weight, type of diagnosed disease, stage and grade, treatment, co-medication, co-morbidities and response to treatment (CP) or data on their sex, age, weight and ethnicity (HV). The demographic characteristics of the HV and CP in this study are listed in **Table 1**.

Assessment of toxicity in patients undergoing anticancer treatment

Toxicity after the first cycle of chemotherapy or the first course of radiotherapy or concurrent radiotherapy and chemotherapy was graded according to The National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (CTCAE). Assessment of neutropenia or other myelotoxicity was based upon blood analysis before second cycle of treatment. The analysis of toxicity was based on a comparison of the rates of grade 3 and greater toxicity.

Genotyping

DNA was isolated directly from blood using Blood Mini Kit (Qiagen, UK) according to the manufacturer's instructions and genotyped for T2444C SNP by pyrosequencing using the PSQ96 system (Pyrosequencing, Uppsala, Sweden) and for CA microsatellite capillary using the electrophoresis system CEQ8000 (Beckman Coulter, UK) as described previously [20].

Western blot analysis

Briefly, the cell pellet was lysed in 100 μ l of Laemmli buffer with 1x Halt protease inhibitor cocktail (Thermo Fisher Scientific) sonicated on ice for 10 s and heated in loading dye containing β -mercaptoethanol and bromophenol blue at 95°C for 5 min. Lysates (30 μ g of protein per lane) were run on Tris-HCl 5-20% polyacrylamide gels (Bio-Rad, Hercules, CA)

along with purified recombinant PARP-1 immunoblotting standard (0-40 ng: Enzo Life Sciences, UK) at 100 V for 2 h and transferred for 1 h at 4°C into a nitrocellulose membrane (Hybond-C, Amersham, UK) (Criterion electrophoresis and blotting apparatus, Bio-Rad). After blocking for 1 h in PBS-MT (PBS+ 5% non-fat powdered dried milk and 0.5% Tween 20) the membrane was incubated overnight at 4°C with an anti-PARP-1 C2-10 primary antibody (1:2000 in PBS-MT Trevigen, Gaithersburg, MD) washed 3x in PBS-T (PBS+ 0.5% Tween 20), and then incubated with the HRP-linked secondary goat anti-mouse antibody (1:1000 in PBS-MT Dako) washed again for 1 h in PBS-T and dried. The protein was visualised with ECL plus detection kit (Amersham, Little Chalford, UK) using the manufacturer's protocol followed by chemiluminescence detection using a Fuji LAS3000 with imaging software (Fuji LAS Image version 1.1, Raytek). PARP-1 expression was quantified by reference to the recombinant PARP-1 standard curve. This assay was validated to GCLP standard for evaluation of patient samples (E. Mulligan and T. Zaremba, unpublished data). Validation studies showed that loading the lysate in duplicate with protein determination gave more reliable data than use of a loading control such as GAPDH and β -actin. Additionally, using a purified PARP-1 standard and a quality control sample (protein extract from K562 cells) assured the quality of transfer and allowed the most precise protein quantification.

PARP activity assay

Total stimutable PARP activity was measured by modification of a previously described method [6] validated to GCLP standard and used as a pharmacodynamic endpoint for clinical trials [18]. This assay measures PARP activity that has been maximally stimulated by a double-stranded oligonucleotide in the presence of excess NAD^+ , thereby eliminating error due to variable activation of the enzyme by DNA damage accidentally introduced during processing. Quality control (QC) samples of L1210 cells were included in each assay. As part of the validation of this assay the day-to-day variation between samples from the same individual was measured in independent experiments. In 8 individual HV the mean maximum variation in PARP activity, measured on 3 different days, was 1.5 ± 0.2 -fold. PARP activity was measured in triplicate samples of 10^4 digitonin permeabilised cells in a reaction mixture containing 350 $\mu\text{mol/L}$ NAD^+ and 10 $\mu\text{g/mL}$ oligonucleotide (CGGAATTCCG) (Europrim, Invitrogen, UK) in a reaction buffer of 100 mmol/L Tris-HCl, 120 mmol/L MgCl_2 (pH 7.8) in a final volume of 100 μL for 6 min at 26°C. After blotting onto a nitrocellulose membrane (Hybond-N, Amersham, UK), the poly(ADP-ribose) was detected following incubation with the primary anti-PAR 10H antibody 1:1000 then HRP-conjugated goat anti-mouse secondary antibody (1:1000 Dako, Ely, UK) and finally ECL reaction and chemiluminescence detection as described above. Results were expressed relative to the number of cells loaded by reference to a poly(ADP-ribose) standard curve (0-25 pmol: Enzo Life Sciences).

Mouse liver samples were thawed and the wet weight recorded prior to homogenisation in 3 volumes ice-cold isotonic buffer (Ultra-Turrax T25, Janke and Kunkel, Staufen, Germany). The homogenate was diluted with isotonic buffer to yield a final dilution of 1 in 2000. The protein content was measured by the colorimetric Pierce protein assay (Thermo Scientific) prior to assay of PARP activity as described above.

Statistical analysis

Each sample was analyzed in triplicate (PARP activity) or in duplicate (PARP-1 expression) and mean presented. The normality of the data distribution was tested by Shapiro-Wilk and D'Agostino & Pearson tests (GraphPad, La Jolla, CA). The distribution of PARP activity and expression was highly skewed and so a base-10 logarithmic transformation was applied in order to obtain a more Gaussian like distribution. Mean log transformed PARP activity, and expression, was compared between sexes and between HV and CP using the Student's t-test. Analysis of covariance (ANCOVA) and linear regression were used to determine associations between activity and expression, and between activity, expression and age,

weight and sex. The χ^2 test and Freeman-Halton extension of the Fisher's exact test were used for analysis of genotype frequencies. The level of significance was set at 0.05.

RESULTS

PARP-1 gene polymorphisms

T2444C SNP (Val762Ala) in the catalytic domain

The T2444C SNP (rs1136410), resulting in an amino acid substitution, Val762Ala, in the PARP-1 catalytic domain is reported to lead to reduced PARP-1 catalytic activity. The genotype frequencies are given in **Table 2**. There was no evidence that the distribution was not in Hardy-Weinberg equilibrium in HV and CP ($P = 0.88$ and $P = 0.31$, respectively). The variant (minor) allele frequency (MAF) for both groups was 14% and was within reported range for studied populations (5 - 33%) [21-23]. There was no difference in genotype distribution between HV and CP ($P = 0.9$). Neither the T/C nor the C/C genotypes were associated with an increased risk of cancer when compared with T/T (OR, 1.06; 95% CI, 0.50 – 2.20 and 0.48; 95% CI, 0.03 – 7.81, respectively).

(CA)_n microsatellite instability in *PARP-1* promoter region

It has been proposed that a long CA polymorphism in the promoter region of the *PARP-1* gene may result in increased PARP-1 expression. We therefore investigated the length of these microsatellite repeats in all subjects' genomic DNA samples. Analysis of the allele frequencies in HV and CP revealed the presence of the two most common alleles namely (CA)₁₁ and (CA)₁₅, which formed the three most common genotypes: HV, (CA)₁₁/(CA)₁₁ (61%), (CA)₁₁/(CA)₁₅ (16%) and (CA)₁₅/(CA)₁₅ (9%) and CP, (CA)₁₁/(CA)₁₁ (78%), (CA)₁₁/(CA)₁₅ (14%) and (CA)₁₅/(CA)₁₅ (3%). As previously established [24] we grouped the CA microsatellite into two alleles: S (Short) comprising (CA)₁₁ – (CA)₁₂ and L (Long) comprising (CA)₁₃ – (CA)₂₀. Genotype frequencies given by this biallelic approach are presented in **Table 2**. There was a statistically significant difference in the genotype distribution between cancer patients and control subjects ($P = 0.003$); CP had a higher frequency of the SS genotype compared to HV (80% versus 59%). The SS genotype was significantly associated with an increased risk of cancer (OR, 5.22; 95% CI, 1.79–15.24) using LL as the reference group.

PARP-1 expression

We found that PARP-1 protein expression (see for example, **Supplementary Figure 1S**), successfully analyzed in 44 HV subjects, showed a large variation between the lowest and the highest expression in subjects (CV = 95%); range 0.02 - 0.78 ng PARP-1/μg protein with a mean value of 0.21 ng/μg and a median value of 0.12 ng/μg. Significant variation in PARP-1 expression was also observed in CP (0.03 - 1.55 ng/μg, CV = 104%) with a mean value of 0.23 ng/μg and median value of 0.16 ng/μg. We did not observe any statistically significant difference in expression between HV and CP ($P = 0.18$, **Fig. 1A**) or men and women either in HV or in CP ($P = 0.1$ and $P = 0.13$, respectively, **Fig. 1B**).

PARP activity

There was a large variation in PARP activity (see for example, **Supplementary Fig. 2S**) in HV ($n = 56$) with values ranging between 10 and 2190 pmol PAR/10⁶ PBMCs (CV = 120%), mean value of 508.6 pmol/10⁶ cells and median value of 260 pmol PAR/10⁶ cells. Similarly, we observed large variation in PARP activity between CP ($n = 118$, CV = 137%) ranging between 10-2600 pmol/10⁶ cells with mean value of 357.7 pmol/10⁶ cells and median value of 160 pmol PAR/10⁶ cells. There was no statistically significant difference in PARP activity between HV and CP ($P = 0.45$, **Fig. 2A**). However, we observed a difference in PARP activity in HV between men and women ($P = 0.04$, **Fig. 2B**).

Dependence of PARP activity on *PARP-1* genotype and PARP-1 protein expression

We found no association between the T244C genotype and PARP activity (T/T vs. T/C, $P = 0.24$ and $P = 0.34$, for HV and CP respectively, **Fig. 3A**). It was not possible to perform any statistical analysis on the C/C genotype as we only had one sample with the C/C variant genotype in each group of subjects. We found no association between the (CA)_n microsatellite polymorphism and PARP-1 expression (**Fig. 3B**).

We hypothesised that PARP activity would be dependent on the level of PARP-1 protein expression. There was a statistically significant but modest positive correlation between the level of PARP-1 protein expression and PARP activity in the HV ($n = 44$, $R^2 = 0.19$, $P = 0.003$, **Fig. 4A**). A positive but even weaker correlation was also found between PARP-1 expression and activity in CP ($n = 118$, $R^2 = 0.06$, $P = 0.01$, **Fig. 4B**).

Demographic effects on PARP expression and activity

In contrast to PARP-1 expression, comparison of gender differences in PARP activity in HV (**Fig. 2B**) revealed that men had significantly higher activity than women ($P = 0.04$). The median PARP activity in PBMCs from men was 550 pmol PAR/10⁶ cells (range 10 – 1700 pmol PAR/10⁶ cells) with CV = 83.7%. The median value for women was 130 pmol PAR/10⁶ cells (range 10 – 21900 pmol PAR/10⁶ cells) with CV = 147.4%. The gender difference persisted after allowing for differences in PARP-1 expression (ANCOVA). On average, males had a 1.9 fold (95% CI, 1.2 - 2.8) higher level of PARP activity compared to females after adjusting for differences in PARP-1 expression ($P = 0.006$).

Following the previous reports showing that the age of the subject may affect PARP activity we investigated the relationship between the age and weight of subjects and PARP-1 expression and activity. PARP activity was negatively correlated with age ($P = 0.02$) in CP but a similar association was not seen in HV ($P = 0.9$) (**Fig. 5A**). On average, for each 10 year increase in age, PARP activity in CP reduced by 19% (95% CI, 4 - 31%). PARP activity was not associated with weight in HV or CP (**Supplementary Figure 3S**). We observed no association between PARP-1 expression and age (**Fig. 5B**), however PARP-1 expression was found to be significantly positively correlated with weight in CP ($P = 0.01$). On average, for each 10 kg increase in weight, PARP-1 expression increased by 13% (95% CI, 3 - 23%). This association was not found in HV (**Supplementary Figure 4S**).

In vivo studies of PARP activity in mice treated with estrogen

To investigate the role of estrogen in the regulation of PARP activity we performed hormonal manipulation in mice. PARP activity in PBMCs was approximately 40% higher in male mice (920 ± 20 pmol PAR/10⁶ cells) compared with female mice (570 ± 70 pmol PAR/10⁶ cells, $P = 0.004$) (**Fig. 6A**). Castration led to a significant decrease in PARP activity (710 ± 38 pmol PAR/10⁶ cells) ($P = 0.0005$). Estrogen supplementation of castrated male mice did not change the level of PARP activity. Paradoxically, estrogen supplementation in female mice caused a significant increase in PARP activity in PBMCs (880 ± 30 pmol PAR/10⁶ cells, $P = 0.003$) bringing it to the level similar to that in control untreated male mice. In marked contrast, PARP activity was about 80% reduced in liver homogenates from estrogen treated female mice (0.15 ± 0.05 pmol PAR/mg protein) compared with untreated female mice (0.9 ± 0.42 pmol PAR/mg protein) (**Fig. 6B**).

PARP activity and patients' response to treatment

We evaluated patients' response to anticancer treatment in terms of toxicity in 44 patients. We have only chosen patients whose treatment was "PARP relevant" that is, those agents known to cause more cytotoxicity and toxicity in PARP null or inhibited cells or mice, respectively. We studied patients treated with temozolomide and dacarbazine (alkylating agents), radiotherapy only (ionizing radiation), and radiotherapy in combination with the following chemotherapeutic agents: temozolomide, cisplatin (radiosensitizer, cross-linking agent), capecitabine (antimetabolite). Among the 44 patients studied 15 (34%) developed

toxicity grade 3 or greater (**Table 3**). The remaining 29 patients (66%) tolerated treatment well with no toxicity or toxicity less than grade 3 (**Supplementary Table 3S**). Patients treated with radiotherapy in combination with cisplatin were far more likely to experience high grade 3 or greater toxicity, OR 13.2; 95% CI, 2.9 - 58.9. However, even after adjusting for exposure to cisplatin, there was no evidence of a relationship between high grade toxicity and PARP activity or PARP-1 expression ($P = 0.5$ and $P = 0.74$, respectively), nor was there evidence of a relationship between high grade toxicity and T2444C genotype ($P = 0.22$).

DISCUSSION

Since PARP activity may have profound implications for health and studies reveal a wide inter-individual variation in PARP activity the overall goal of this study was to determine the mechanisms underlying this inter-individual variation. To this end we measured polymorphisms in the *PARP-1* gene that could affect its expression and activity, PARP-1 protein levels and demographic factors in relation to PARP activity in PBMCs from human subjects.

Our study revealed a large variation (CV = 120% and 137% for HV and CP, respectively) in PARP activity between individuals. Inter-individual variation in PARP-1 expression was much lower (CV = 95% and 104% for HV and CP, respectively). Although there was a positive correlation between PARP-1 expression and activity in the HV and CP, supporting the hypothesis that PARP activity reflects its abundance, the correlation was not as strong as expected with only about 20% (HV) and <10% (CP) of the variation in activity is explained by variation in expression.

In the 174 individuals we studied we did not find that the T2444C SNP in the active site affected PARP activity, which is similar to observations made by Cottet et al. [21] in 95 individuals. However, another study [4] of 354 individuals as well as *in vitro* studies [13, 15] using purified PARP enzyme did report a decrease in PARP activity associated with the variant allele. Whether the difference is only detectable in large studies or whether the method used to determine PARP activity (H_2O_2 stimulation [4] or *in vitro* analysis [13, 15]) is responsible for the different findings is not possible to say at this time, but it is clearly worthy of further investigation using a standardised protocol. Similarly, the observed inter-individual variation in PARP-1 expression was not affected by the polymorphisms in the promoter region of *PARP-1* gene; the two allelic approach, grouping all identified alleles into short (S) and (L) and further correlation analysis with PARP-1 expression did not show any association between the level of PARP-1 protein and the length of microsatellite.

The lack of correlation between PARP activity and genotype, with only a limited influence of PARP-1 expression, suggested that other factors may play a role in the regulation of PARP activity, such as demographics. We confirmed previously reported [16] negative correlation between PARP activity and age. This was only seen in CP where there was a 66-year difference between the youngest and oldest subject (22-88 years), although there was substantial overlap in the age distribution in the HV compared to the CP the age distribution was narrower (41 years; 18-69) and hence a trend more difficult to determine. For the first time we showed that PARP activity was not associated with weight.

Our most striking and novel observation was a statistically significant difference ($P = 0.04$) in PARP activity between men and women (HV). We found that younger women (<45 years, an age chosen based on epidemiological studies: www.nhs.uk/Conditions/Menopause) generally had lower activity (mean 414.1 pmol PAR/10⁶ cells, 95% CI, 133.1 - 695.1) than older women, who had intermediate activity (mean 427.5 pmol PAR/10⁶ cells, 95% CI, 73.6 - 781.3) between young women and men (mean 688.8 pmol PAR/10⁶ cells, 95% CI, 419.1 - 958.4) but the difference was not statistically significant. The gender differences found in HV were not found in CP ($P = 0.242$), possibly because they were largely in the over 45 year-old group (94% of CP women). There are no previous reports of gender differences in PARP activity in humans although sexual dimorphisms in PARP-1 activity have been reported in animal models [25, 26]. Our finding in humans and published data in animals suggesting hormonal regulation of PARP activity led us to conduct further investigations of hormone effects in mice. As with the human subjects PARP activity in male mice was significantly

higher than in females. PARP activity in castrated males was significantly reduced compared to intact males and similar to that in females. However, estrogen supplementation failed to reduce PARP activity further in castrated males. Thus, it seems more likely that gender differences in PARP activity in PBMCs are due to androgen-mediated stimulation rather than estrogen-mediated inhibition of PARP activity. Consistent with this hypothesis is the observation that PAR formation was approx. 2-fold higher in brain tissue from male mice than females in a stroke model [27] and that PARP-1 mediated damage following cerebral ischaemia was significantly reduced in castrated mice [28]. However, estrogen supplementation profoundly reduced PARP activity in the liver, consistent with the observed estrogen-mediated PARP inhibition in the livers but not PBMCs of male mice treated with LPS [25]. This differential effect of estrogen on different tissues warrants further investigation as it may be relevant to diseases where PARP-1 activation has pathological effects (e.g. diabetes, [29]) and where there are sex differences in incidence or outcome (e.g. primary liver cancer, [30]).

One of the aims of this study was to correlate PARP activity with patients' response to treatment in terms of toxicity. The work presented here is the first study of PARP activity in relation to toxicity in patients receiving chemo- or radiotherapy. Patients vary in their response to chemo- and/or radiotherapy and the underlying mechanism comprise numerous different factors including the patient's genetic profile (pharmacogenetics) and age. Recent studies demonstrate a potentiation of both anticancer activity and toxicity when cytotoxics are combined with PARP inhibitors [18, 19, 31]. These data suggest that PARP activity may not only have an impact on therapeutic response but also the toxic response to chemo- or radiotherapy. We did not observe any significant difference in PARP activity between patients who suffered substantial toxicity (grade 3 and above) and those with no symptoms of toxicity. We confirmed the high levels of toxicity with cisplatin-radiotherapy combinations [32], but again the patients' PARP activity did not appear to influence the toxic response. Additionally, we observed that 40% of men but only 21% of women experienced high grade toxicity. In contrast to our findings, several clinical trials have reported greater toxicity in women [33, 34].

Analysis of the CA repeat polymorphism and the two allelic approach, where alleles were grouped into short (S) and (L) revealed that the SS genotype was associated with increased risk of cancer (OR, 5.22; 95% CI, 1.79–15.24) compared with LL genotype, which was underrepresented in CP. To the best of our knowledge this is the first time that the length of the CA microsatellite has been linked with cancer risk. Since we did not find an associated effect on PARP-1 expression or activity the functional consequences of short CA repeat that could explain the increased cancer risk remains to be determined.

In summary, in this study we tried to find a possible explanation for the observed large inter-individual variation in PARP activity and if it had an impact on patients' toxicity, with a view to progression towards individualised therapy. We did not find any strong evidence that genetic factors play a role in determining PARP activity. The lack of any association between PARP activity and genotype or sex in CP may indicate additional factors (e.g., stress hormones, interaction with other proteins and posttranslational modifications) may play a role in PARP activation. However, we did not find any association between PARP activity and patients' response to treatment.

An unexpected observation was that PARP activity shows only a modest dependence on PARP-1 protein expression, indicating that endogenous or exogenous factors play a major role in regulating PARP activity. Importantly, we show for the first time gender difference in PARP activity in the normal human population. Given that PARP activity can have implications for human health, further investigations of the role of hormones on PARP activity and the tissue specificity of the effect are warranted.

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PARP expression and activity

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PARP expression and activity

Tables

Table 1 Baseline characteristics of eligible group of participants. For age, and weight mean values \pm SD are given, with range in brackets and median value in square brackets (age only). Information on age was available for n = 156 and for weight n = 139.

Variable	HV	CP
Total	56	118
Sex		
Men	20 (36%)	65 (55%)
Women	36 (64%)	53 (45%)
Ethnic origin		
Caucasian	53 (95%)	118 (100%)
African	1 (2%)	
Asian	2 (3%)	
Age (years)	36 \pm 13 (18-69) [30]	63 \pm 13 (21-88) [64]
Men	31 \pm 12 [26]	62 \pm 14 [65]
Women	39 \pm 13 [40]	61 \pm 12 [60]
Body weight (kg)	68 \pm 11 (48-95)	74 \pm 17 (42-120)
Men	75 \pm 6	81 \pm 17
Women	64 \pm 11	67 \pm 14

Table 2 Distribution of PARP-1 genotypes.

Polymorphisms	Major homozygote T/T, n (%)	Heterozygote T/C, n (%)	Minor Homozygote C/C, n (%)	<i>P</i> =0.9
T2444C (Val762Ala)				
HV	41 (73)	14 (25)	1 (2)	
CP	86 (73)	31 (26)	1 (1)	
OR (95% CI)	Ref.	1.06 (0.50-2.20)	0.48 (0.03-7.81)	
(CA)_n	SS, n (%)	SL, n (%)	LL, n (%)	<i>P</i> =0.003
HV	33 (59)	12 (21)	11 (20)	
CP	94 (80)	18 (15)	6 (5)	
OR (95% CI)	5.22 (1.79-15.24)	2.75 (0.80-9.45)	Ref.	

*For active site T2444C SNP C/C is a variant/variant.

Promoter polymorphism SS – short alleles (CA)₁₁₋₁₂/(CA)₁₁₋₁₂, SL – short/long alleles (CA)₁₁₋₁₂/(CA)₁₃₋₂₀, LL - long/long alleles (CA)₁₃₋₂₀/(CA)₁₃₋₂₀.

PARP expression and activity

Table 3 High grade toxicity (grade ≥ 3) observed in patients treated with PARP relevant treatment (n = 15). PARP activity expressed as pmol PAR/10⁶ PBMCs. PARP-1 expression presented in ng/ μ g total protein. Genotype for catalytic domain SNP (T2444C) is also given. *Abbreviations:* TMZ, temozolomide; CIS, cisplatin; CAP, capecitabine.

Patient No.	Treatment	Adverse Event grade ≥ 3	PARP activity	PARP expression	T2444C SNP
1015	TMZ	severe allergic reaction	860	0.36	T/T
1119	radiation 55 Gy	mucositis	140	0.14	T/T
1123	tamoxifen + radiation 45 Gy	erythema, skin reaction	390	0.12	T/C
1148	CIS + radiation 63 Gy	vomiting, nausea	580	0.3	T/T
1135	CIS + radiation 63 Gy	mucositis, skin reaction	140	0.19	T/T
1133	CIS + radiation 63 Gy	mucositis, dysphagia	830	0.18	T/T
1066	CIS + radiation 63 Gy	mucositis, dysphagia, diarrhoea, renal failure	130	0.08	T/T
1073	CIS + radiation 55 Gy	lethargy, dysphagia, mucositis	300	0.1	T/C
1055	CIS + radiation 63 Gy	erythema, mucositis	150	0.63	T/C
1033	CIS + radiation 63 Gy	mucositis, skin reaction toward radiotherapy	080	0.12	T/C
1034	CIS + radiation 63 Gy	mucositis, dysphagia	60	0.17	T/T
1009	CIS + radiation 63 Gy	vomiting, diarrhoea	40	0.43	T/T
1001	CIS + radiation 63 Gy	mucositis, erythema, lymphopenia	130	0.19	T/T
1087	CIS + radiation 63 Gy	mucositis	170	0.08	T/C
1004	CAP + radiation 25 Gy	nausea	90	0.16	T/T

Legend to figures

Fig. 1 A PARP-1 expression (\log_{10} transformation) in PBMCs from healthy volunteers (HV, n = 44) and cancer patients (CP, n = 118). *Each data point*, a single individual; *horizontal line*, mean for the respective groups of samples. The difference between the two groups is not statistically significant ($P = 0.18$ by Student's t-test).

B PARP-1 expression (\log_{10} transformation) in HV men (n = 12), HV women (n = 32) CP men (n = 65) and CP women (n = 53). *Each data point*, a single individual; *horizontal line*, mean for the respective groups of samples. The difference between the two groups is not statistically significant ($P = 0.1$ and $P = 0.13$ for HV and CP, respectively).

Fig. 2 A PARP activity (\log_{10} transformation) in PBMCs from healthy volunteers (HV, n = 56) and cancer patients (CP, n = 118). *Each data point*, a single individual; *horizontal line*, mean for the respective groups of samples. The difference between the two groups is not statistically significant ($P = 0.45$ by Student's t-test).

B PARP-1 activity (\log_{10} transformation) in HV men (n = 20), HV women (n = 36), CP men (n = 65) and CP women (n = 53). *Each data point*, a single individual; *horizontal line*, mean for the respective groups of samples. The difference between HV men and women is statistically significant ($P=0.04$ by Student's t-test).

Fig. 3 A Scatter plot of PARP activity in HV and CP in subjects with T2444C T/T, T/C and C/C genotype, respectively. **B** Scatter plot of PARP-1 expression in HV and CP in subjects with promoter polymorphism: SS – short alleles $(CA)_{11-12}/(CA)_{11-12}$, SL – short/long alleles $(CA)_{11-12}/(CA)_{13-20}$, LL - long/long alleles $(CA)_{13-20}/(CA)_{13-20}$, respectively.

Fig. 4

A Correlation between PARP-1 expression (\log_{10} transformation) and PARP activity (\log_{10} transformation) in HV and **B** in CP.

Fig. 5

A Correlation between age and PARP activity (\log_{10} transformation).

B Correlation between age and PARP-1 expression (\log_{10} transformation).

Fig. 6

A Effect of estrogen (E_2) on the PARP activity in the CD1 males, castrated males and females mice. Blood from 3 (controls) or 5 (treated) animals in each group was pooled and used as a single sample. Values are mean \pm SEM. All P values were significant (Student's t-test).

B Effect of estrogen treatment (E_2) on the PARP activity in the liver homogenates from female mice. PARP activity was measured in triplicates per liver sample. 3 livers per group were used.

PARP expression and activity