Abstract. The aim of the present study was to evaluate the utility of various non-invasive parameters for the prediction of tumor development and animal welfare in a murine xenograft model in male C.B-17 SCID (C.B-Igh-1Pl/IcrTac-Prkdcscid) mice. The study showed that body weight, food and water consumption, and an animal welfare assessment (AWA) protocol revealed marked differences between control and cancer lines as the size of the tumor increased. However, only the AWA protocol was effective in predicting the tumor size and the level of fecal corticosterone metabolites (FCM). FCM levels were, however, negatively-correlated to the AWA score, and the tumor size, both when evaluated on a given day and when accumulated over the entire period. In conclusion, the present study demonstrated that body weight and food and water consumption were negatively-affected as tumor developed but only the animal welfare protocol could be used to predict tumor size.

In 2010, it was estimated that up to 95% of animal studies in cancer research were conducted on mice (1), and murine xenograft models are extensively used to investigate cancer biology and pathology, as well as the efficacy of potential anticancer drugs. However, the welfare of animals is often neglected, and very little information exists on the occurrence of pain and stress in animals used as cancer models. As 65-85% of human patients with cancer report experiencing pain and discomfort (2), it is reasonable to assume that the welfare of the laboratory animals is compromised as tumor progresses (3). Pain and stress are, apart from being a serious ethical concern, a potential source of experimental error, as many physiological, immunological, endocrine and behavioral parameters may change as a consequence of such conditions (4-7). Furthermore, as chronic stress by itself may affect the outcome of cancer development (8-11), it is important to control all aspects of unnecessary suffering and to gain further insight on the stress and pain response in animals used as cancer models.

Previous studies on mice and rats have demonstrated behavioral responses indicating suffering in relation to cancer development. However, the response differs markedly from the response seen in other pain-associated models such as after surgery, and it has not yet been possible to reduce the adverse condition suffered by animals used in cancer models (12). This is perhaps due to the multifactorial and complicated causes of suffering in relation to cancer development. In humans, pain secondary to the cancer itself or to its treatment is prominent compared to the pain caused by actual tumor growth (13) and common cancer symptoms such as fatigue, nausea, and constipation (14) may significantly affect the well-being of the patients. Furthermore, chronic pain and stress may be difficult to recognize and estimate as the onset is slow and the intensity not constant during disease development (15). However, despite the challenges in estimating and alleviating suffering in this field, it is important to continue evaluating new methods in order to improve the welfare of the animals. Only when our knowledge increases within this field, will it be possible to determine valid humane end-points and thereby reduce unnecessary suffering of the involved animals.

Combining behavioral observations with quantitative clinical measures, such as body weight and food and water consumption, has provided us with reliable information about
the physiological and psychological state of the animal in several animal models and research settings (16-21). In addition to behavioral observations and clinical measures, the level of fecal corticosterone metabolites (FCM) is increasingly being used as a non-invasive means of assessing pain and stress in various animal species (22). FCM quantification represents many advantages compared to blood measurement of stress hormones. It is generally accepted that FCM represent an integrated amount of these steroids over time and results are unlikely to be biased by the sampling technique, since the technique is non-invasive and does not involve interaction with the animal during the period of interest. Several studies have demonstrated a good correlation between stimulation of the hypothalamic pituitary-adrenal (HPA) axis and fecal glucocorticoid metabolite levels in rats (23-25), companion and farm animals (26-28), poultry (29), non-human primates (30, 31), and several wildlife species (32). In mice, the use of FCM as a reliable parameter of HPA-axis activity has been validated (33) but its reliability in clinical situations has not, however, been fully-elicited (21) and no previous studies have, to our knowledge, measured the response to cancer development. In humans, some cancer types disrupt the circadian pattern of cortisol concentrations in the circulation and increase the basal hormone levels (34). To our knowledge, only one study has investigated the stress hormone levels in response to tumor development in mice (35). The study by Hilf et al., demonstrated that a sarcoma cancer line in mice resulted in an increase in both adrenal and plasma corticosterone, but the adrenal activity differed at various stages of the tumor growth. In guinea pigs, transplantation of leukemia cells resulted in an increase in corticoid production (36) and measurements of FCM may, therefore, be a valuable tool in assessing stress in animal models of cancer and assist in the development of humane end-points.

The aim of the current study was to assess the pain and stress caused by the growth of a subcutaneous prostate cancer xenografts in laboratory mice, using body weight, food and water consumption, FCM and an animal welfare assessment protocol (AWA) as parameters of welfare, and to investigate if any of these parameters would be indicative of tumor development. The AWA score may be of particular benefit in cancer models as it combines various parameters that can easily be assessed by the observer and provides up to-the-minute information on the animal welfare.

Prostate cancer is one of the major cancer types in human males. Pre-clinical research aims at establishing the most clinically relevant prostate cancer models mimicking human prostate cancer disease progression. The predominant cancer cell lines used in this pre-clinical field are LNCaP, PC3 and DU-145 (37). In the present study we investigated the impact on animal welfare of a Sigma Aldrich-derived LNCaP cell line (wild-type) and a genetically modified LNCaP cell line. Materials and Methods

The animal experiments performed in this study were approved by the Animal Experiments Inspectorate under the Danish Ministry of Justice (license number 2011/561-1956). All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (38).

All data achieved in the study were either already existing data achieved in a related xenograft study, using the same animals, or the data were non-invasive observations made on the xenograft mice without extra experimentation on the animals. This serves to fulfill the 3R principles of animal experimentation, mainly refinement and reduction. The prostate xenograft study was a titration study to optimize the number of cells to be inoculated per mouse to achieve a take rate and a growth rate suitable for xenograft cancer efficacy studies.

Animals and housing conditions. Forty-four C.B-17 SCID (C.B-Igh-1/IcrTac-Prkdax8-id) male mice were obtained from Taconic, Ry, Denmark. The animals were allowed to acclimatize for at least one week before the study. The mice were 6-8 weeks of age, weighing 20.5±1.5 g (mean±standard deviation (SD)), at the time of inoculation. The mice were group-housed, with 4-6 mice in each IVC Macrolon cages type II (Tecniplast, Varese, Italy). Food pellets (Altromin 1324; Lage, Germany) and UV-sterilized water were provided ad libitum. Wooden chips (Jelu, J.Ehrler GmbH & Co KG, Rosenberg, Germany) were used as bedding. Bite bricks (Tapvei®), Kortteinen, Finland), PM90/L/R Tapvei nesting material (Tapvei®) and a mouse hut (Lillico, Brogaarden, Gentofte, Denmark) were provided as environmental enrichment. Room temperature was maintained at 20-24°C, air humidity was 55% +/- 10% and the air was changed approximately 12-times per hour. The light was regulated with a 12/12 hour dark/artificial light cycle with light period starting at 6:00 am.

Study design. Four mice were used as control animals. These animals were subjected to the same handling and monitoring as the other mice, but received no injection with cancer cells. Unmodified LNCaP cells (wild-type) and genetically modified LNCaP cells were cultured in standard RPMI culture medium and inoculated subcutaneously into the flank area of the C.B-17 SCID mice. Ten mice were inoculated with 5×10^6 wild-type LNCaP cells (group 1) and ten mice with 1×10^7 wild-type LNCaP cells (group 2). Furthermore, ten mice were inoculated with 5×10^6 genetically modified LNCaP cells (group 3) and ten mice with 1×10^7 genetically modified LNCaP cells (group 4). Cancer cells were inoculated on day 0 (one injection per mouse).

The pain and stress monitoring consisted of the following observations: body weight (individual), food and water consumption (group measurement), AWA (individual), FCM (group measurement), and tumor size (individual) twice a week throughout the study. The animals were euthanized at the end of the study or when reaching humane end-points. The humane end-points were based on an individual assessment of negatively-affected well-being (as assessed by a veterinarian), tumor size greater than 15×15×7.5 mm (which in general will result in a tumor weight of maximum 5% of the animal’s body weight), maximum 20% body weight loss or ulceration of the tumor.

Data collection. Data from all animals were obtained between 9 and 12 am twice a week throughout the study. AWA measurements were obtained by inspecting each mouse before the
The cage was moved from the IVC system and again after the cage was removed and the lid was lifted. Each animal was scored according to Table I. The food and water consumption was calculated by subtracting the measured weight of food or water from the amount measured on the previous occasion. The food and water measurements are presented as individual consumption g/day × number of mice in the given period (n). At the time of weighing, all bedding was removed from each cage and frozen at −21˚C until feces were separated from bedding. The nesting material and mouse hut were re-used during the entire period for each cage to maintain individual olfactory signals within the environment and thereby cause minimal stress in relation to bedding change. FCM were quantified in general as described previously by Sundbom et al. (19). In brief, corticosterone metabolites were extracted by incubating feces in 96% ethanol overnight. In the present study, we used a ratio of 3 ml ethanol per gram feces. Corticosterone levels were analyzed in duplicate using DRG-Diagnostics corticosterone competitive enzyme-linked immunosorbent assay (ELISA) (EIA-4164; DRG Instruments GmbH, Marburg, Germany), in accordance with the manufacturer’s instructions. Standards included in the kit were replaced with a custom nine-point standard curve prepared in 96% ethanol from analytical grade corticosterone (46148; Sigma-Aldrich, St Louis MO, USA) in concentrations spanning from a range between 50 and 0.19 ng/ml. The kit has been verified to have a cross-reactivity equivalent to 7.4% with progesterone, 3.4% with deoxycorticosterone, 1.6 % with 11-dehydrocorticosterone, 0.3% with cortisol and pregnenolone and <0.1% with other steroids. The analytical sensitivity is reported as being less than 1.6 nmol/l. The absorbences were recorded at 450 nm using a Thermo Multiskan Ex microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Results are presented as ng FCM/day × n.

In vivo tumor size was recorded when the tumor was palpable (~1 mm³) and then twice per week until termination, according to the xenograft study protocols. The tumor diameters were measured in two dimensions using a digital slide gauge (Mitutoyo Absolute Digimatic; Aurora, IL, USA) and the volume was estimated by the following equation: L × W × ½W (length × width × ½ width). At termination the weight of the tumor was measured after being removed from the animal.

Statistical analysis. Relative weight loss was analyzed by a mixed linear model including a tumor type-specific fixed effect of time, and tumor type-specific trends of tumor volume, FCM, water consumption, start weight, and food consumption. Mouse and replication groups were included in the model as random effects. Model reduction was performed with backwards elimination based on F-tests with a 5% cut-off value.

AWA scores were analyzed by robust least squares methodology, including a tumor type-specific effect of time, and tumor type-specific trends of tumor volume, FCM, water consumption, starting weight, and food consumption. This corresponds to a linear normal generalized estimating equation (GEE) model with working independence correlation structure on the replication group level. Model reduction was performed with backwards elimination based on robust generalized score tests with a 5% cut-off value.

The GEE approach described above was also used to analyze tumor volume including a tumor type-specific effect of time, and tumor type-specific trends of FCM and starting weight. Food and

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Table I. Animal welfare assessment protocol used to assess the welfare of each individual animal twice a week throughout the study period.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Score ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Normal *</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>General lack of grooming*</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fresh ocular and nasal discharges *</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Bloodstained or mucopurulent discharge from any orifice *</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>* Adjust score by an extra point if: Tightening of the eyes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Adjust score by an extra point if: Ears pulling apart and back from baseline</td>
<td>3</td>
</tr>
<tr>
<td>Movement and body posture</td>
<td>Normal and upright movement and posture</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Less mobile, lying still but runs of when touched</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hunched up, unable to maintain an upright position, sitting still, unwilling to move</td>
<td>3</td>
</tr>
<tr>
<td>Natural behaviour</td>
<td>Interacts with peers, awake, active and responding to surroundings, no self-mutilation</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Little peer interaction, less mobile and isolated, but alert, no self-mutilation</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>No peer interaction, restless or very still, buries/hides, not alert, self-mutilation</td>
<td>3</td>
</tr>
<tr>
<td>Fur quality</td>
<td>Smooth shinning fur with no or only focal areas of mild piloerection</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mild generalized piloerection</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Marked generalized piloerection, possible staining of the fur</td>
<td>3</td>
</tr>
<tr>
<td>Respiration</td>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Slightly labored or decreased breathing</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Severe changes in respiration pattern and frequency</td>
<td>3</td>
</tr>
<tr>
<td>Tumor</td>
<td>Smooth tumor</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Irregular surface of tumor and/or invasive tumor and/or skin covering tumor is reddish, stretched and almost cracking</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Ulcerated tumor</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>0-20</td>
</tr>
</tbody>
</table>
water consumption were analyzed similarly replacing start weight by average starting weight within the replication group.

The final tumor weight was analyzed by a mixed linear model including fixed main effects of tumor type, individual starting weight, cumulative FCM, food consumption, and water consumption per animal within replication group, as well as a random effect of replication group. Model reduction was performed with backwards elimination based on F-tests with a 5% cut-off value.

All analyses were performed using SAS version 9.1.3 (SAS Institute, Cary, NC) and the statistical programming environment R (www.r-project.org).

**Results**

The body weight, food and water consumption and FCM levels during the study period are shown in Figure 1.

Relative weight loss was significantly affected by cancer type, with the effect being modified over time ($p=0.01$), tumor volume ($p<0.0001$), water consumption modified by cancer type ($p=0.003$), and food consumption modified by cancer type ($p=0.002$). Figure 2 shows model-based profiles of relative weight loss for the four cancer types.
AWA scores were significantly affected by tumor volume \( (p=0.006) \) and FCM \( (p=0.05) \). The change in AWA score per unit increase in FCM on a given day, with a fixed tumor volume, was estimated to be \(-0.05\) with a 95% confidence interval (CI) of \(-0.09\) to \(-0.01\).

A statistically significant effect of FCM was seen on tumor volume \( (p=0.02) \), food consumption \( (p=0.01) \), and water consumption \( (p=0.01) \). The final tumor weight was significantly affected by cumulative FCM per animal within the replication group \( (p=0.006) \). Table II summarizes the estimated trends and 95% CI of the trends between FCM and the above-mentioned parameters.

All analyses were repeated replacing FCM with the FCM/g feces, and cumulative FCM with the ratio between cumulative FCM and cumulative feces. In these analyses, none of the associations reported above were detected (data not shown).

**Discussion**

In 1988, the first edition of the United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Guidelines on In Vivo Cancer Models was published (39). This document included recommendations on tumor burden, duration of tests, maximally permitted body weight loss and general recommendation on the use of humane end-points. The latest edition of the guidelines from 2010 emphasizes that every effort should be made to identifying early humane end-points for the animals used and describes clinical signs that may be useful in evaluating the welfare of the animals (1). However, defining reliable humane end-points and evaluating the state of the animal may be challenging in laboratory mice, mainly due to these animals’ natural behavior as a prey animal in hiding any obvious signs of suffering. Furthermore, it may be difficult to predict the pain and stress response in various cancer models, as the total burden of the procedure is highly affected by cancer type, site of tumor development, degree of metastasis etc. This, combined with the multifactorial cause of suffering in relation to cancer development, makes it beneficial to investigate several parameters combined concerning behavioral, endocrine and clinical changes as indications of the animals’ well-being (40-42). Animal welfare assessment protocols may be valuable tools in this respect, as these can be specifically developed for the individual study protocol (43). This approach has proven especially useful with new procedures or when users are not familiar with the adverse effects of a treatment (44). The animal welfare protocol used in the present study was significantly predictive of the tumor burden: the higher the AWA score, the larger the tumor volume. Surprisingly, the AWA score was negatively correlated with FCM, showing that the higher the FCM level was, the lower the AWA score was. FCM was furthermore negatively-correlated with tumor volume, both at various days and when the cumulative FCM level over the entire period was considered and compared to the weight of the tumor at the end of the study. This was contrary to what was expected, i.e. that the FCM would increase in response to increased suffering as the tumor progressed. However, as a chronically elevated glucocorticoid level may have several unwanted health consequences (4), high levels of circulating corticosterone exhibit negative feedback on the pituitary in order to maintain normal homeostasis (45). The negative feedback or neuroendocrine dysregulation may be the cause of the negative relationship between FCM and tumor

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Significance (p-value)</th>
<th>Estimated trend per ng FCM</th>
<th>95% CI of trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food consumption</td>
<td>0.01</td>
<td>0.04 g</td>
<td>0.03 - 0.05</td>
</tr>
<tr>
<td>Water consumption</td>
<td>0.01</td>
<td>0.03 g</td>
<td>0.03 - 0.04</td>
</tr>
<tr>
<td>AWA-score</td>
<td>0.05</td>
<td>-0.05</td>
<td>-0.09 - -0.01</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>0.02</td>
<td>-15.75 mm^3</td>
<td>-25.47 - -6.04</td>
</tr>
<tr>
<td>Final tumor weight</td>
<td>0.006</td>
<td>-0.003 g</td>
<td>-0.006 - -0.001</td>
</tr>
</tbody>
</table>
progression in this study. Another explanation could be changes in the enzymes metabolizing corticosterone during cancer development, thereby masking the actual level of corticosterone in the blood. In tumor-bearing rats, it was demonstrated that the metabolism of exogenous cortisol increased and the plasma binding of cortisol decreased compared to control animals (46). The change in metabolism of cortisol led to an increase in the metabolite 6-OH-cortisol measured both in in vivo and in vitro experiments (46). 6-OH-Cortisol is a polar, water-soluble compound excreted primarily in the urine (47). As alterations in the fractions of metabolites of cortisol have been demonstrated in humans with cancer (48), a similar change of endogenous corticosterone metabolites could be expected in tumor-bearing mice. As the assay used in the present study measured the level of native corticosterone, as well as some metabolites of corticosterone, changes in metabolite composition may not be detectable in the present assay. Furthermore, an increase in urinary excretion of corticosterone metabolites would not be detected. This illustrates some of the problems of interpreting FCM as an indicator of the serum glucocorticoid levels. In addition, the primary glucocorticoid corticosterone is almost absent from stool and mainly metabolites are present (49), and it has not yet been fully elucidated how the various metabolites reflect the actual HPA axis activation, particularly in mice. Further studies are, therefore, needed to determine how the endogenous levels of corticosterone change during cancer development and how the tumor development affects the metabolism and excretion of the hormone. Based on this, FCM seem not to be a reliable indicator of HPA axis activity in mice in the present setup, and interpretation of FCM as an indicator of preceding stress levels in cancer models may be misleading.

Statistically significant relationships were found between FCM and food and water consumption. Mice having high levels of FCM ate and drank more than mice with low FCM levels did. This again illustrates that FCM excretion does not seem useful as an indicator of the animals’ well-being in the present model, as food and water consumption decreased throughout the study period in the tumor-bearing mice. Food and water consumption have been proven to be useful indicators of pain in relation to surgery (42), and reduced consumption is a general non-specific sign of sickness in mice (50). The reduced food and water consumption may account for some of the decrease observed in body weight for the tumor-bearing mice. Reduced consumption combined with a marked decrease in body weight is likely to have significantly negative consequences for the animals as this may be involved in developing cachexia, malnutrition and eventually, morbidity and mortality (51). The marked decrease in body weight did not directly predict tumor volume, but only through the other parameters such as food and water consumption and FCM levels. The decrease in body weight may, thus, not be a consequence of the growing tumor alone, which is in agreement with other studies demonstrating that loss of body condition due to cancer is due to multiple and not fully-elicited causes [for review see (52)].

In conclusion, the present study demonstrated that the cancer cell lines caused significantly impaired animal welfare, illustrated by decreased body weight, food and water consumption and an elevated AWA score. Furthermore, the study showed that FCM levels were negatively-correlated with tumor size, both in regard to the level on specific days and the cumulative value over the entire period. The animal welfare protocol used was effective in differentiating the mice inoculated with cancer cells from control mice, but had overall low correlation with any of the parameters investigated except the tumor volume and FCM. Overall, the data give evidence that the two prostate cancer cell lines used caused changes in clinical, behavioral and endocrine parameters and that the AWA score developed for the present study, was predictive of the tumor size. The AWA score may, thus, be a valuable tool as a humane end-point, whereas FCM does not seem suitable for this purpose in the present animal model.

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