Research Article

Tumour growth in mice resistant to diet-induced obesity

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Abstract

Obesity is a chronic disease with associated increases in the incidence, and a reduction in survival, of many cancer types. Obesity results from an imbalance in calorie intake and calorie requirement. This study aimed to investigate the separate effects of high-fat diet and obesity on cancer in an animal model resistant to diet-induced obesity. Male BALB/c mice fed long-term on a high-fat, Western-style diet were implanted with syngeneic CT26 colon adenocarcinoma cells and compared to mice fed normal diet. BALB/c mice on high-fat diet were 10% heavier than mice fed normal diet, with no difference in tumour growth rates or tumour cell proliferation. Subgroups of mice that became obese on high-fat diet, however, showed increased tumour growth rates compared to mice fed normal diet, whereas mice that remained slim showed no difference in tumour growth. Protein arrays identified several adipokines that were expressed at different levels in the subgroups, including serum Tissue Inhibitors of Metallo-Proteinases (TIMP-1) and tumour C-Reactive Protein (CRP). In conclusion, tumour growth was enhanced in mice unable to resist obesity, and adipokine profiles were affected by the animals' ability to resist obesity.

Introduction

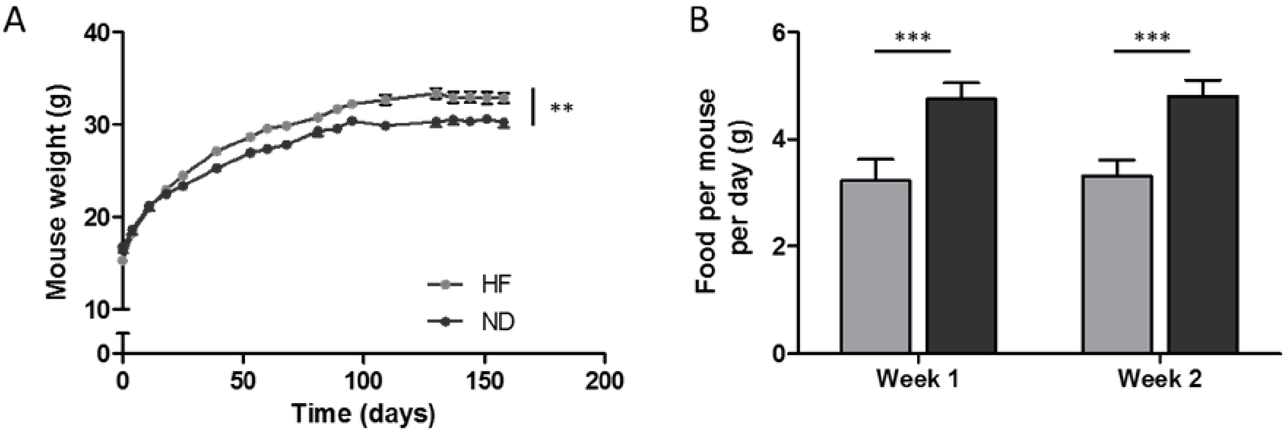
There is convincing epidemiological evidence that body fatness is a strong risk factor for a range of cancers, including colorectal, breast, pancreatic, endometrial and ovarian, with over 20% of obesity-associated cancers in the US attributable to excess adiposity *per se* (World Cancer Research Fund 2007). Increased adiposity is not only associated with increased cancer incidence, but also with increased mortality and reduced survival following cancer diagnosis (Parekh *et al.* 2012). Specifically, for patients with colorectal cancer, pre-diagnostic body mass index (BMI) is predictive of response to therapy and survival (Campbell *et al.* 2012, Meyerhardt *et al.* 2004). The mechanisms underlying this association are under intense investigation, but have not been resolved. Chronic inflammation is a plausible link between obesity and cancer, but other factors will also be contributing (Park *et al.* 2014).

Obesity results from an imbalance in calorie intake and calorie requirement, and is modulated by genetic predisposition. For this study, obesity-resistant BALB/c mice (Fearnside *et al.* 2008, Montgomery *et al.* 2013, Nishikawa *et al.* 2012, Waller-Evans *et al.* 2013) fed a Western-style, high-fat diet were chosen. These wild type mice are immune-competent, which is essential to study the inflammatory effects of obesity, and thus require syngeneic mouse tumours, such as the CT26 colon adenocarcinoma (Meijer *et al.* 2008). This study aimed to investigate the associations between diet, obesity and cancer risk, and specifically, the separate effects of high fat diet and obesity in an animal model with cancer.

Materials and Methods

Ethics

Approval for this study was obtained from the University of Otago Animal Ethics Committee (ethics ap-



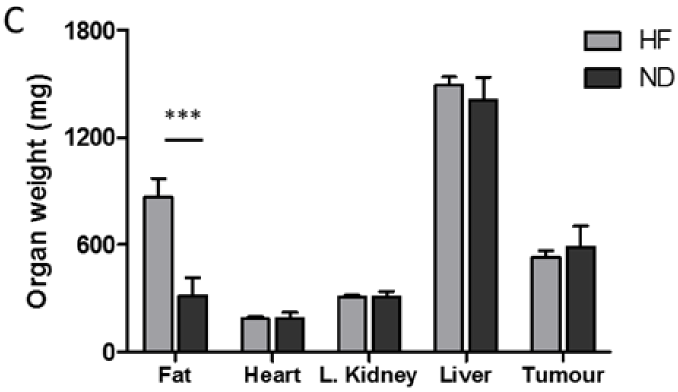


Figure 1. Comparison of mouse weights (A), food consumption (B) and organ weights (C) in mice fed on high fat diet (HF) vs normal diet (ND). (A) Young male BALB/c mice on HF or ND diet were weighed weekly for 5 months, prior to tumour implantation and sacrifice (HF n=31, ND n=10). (B) Daily food consumption over two weeks was analysed in groups of 5 mice each HF and ND. (C) Organs and tumour were weighed (HF n=20, ND n=9). Data were analysed using 2-way ANOVA followed by post-hoc Bonferroni tests; ** $p<0.01$, *** $p<0.001$.

proval number C6/10). International guidelines on animal welfare were strictly followed (Workman *et al.* 2010).

Mouse model

BALB/c mice were bred in-house, and male mice were chosen after weaning. Mice were weighed at least once a week. Due to fighting, male mice were all housed singly for the last 4 months of the study. Mice fed on high fat diet were divided into subgroups according to their final body weight; HFF >35g, HFM 32-35g, HFS <32g.

Diet

Male mice (4 weeks old), following weaning, were randomly divided into two groups, with one of the groups allowed to feed *ad libitum* on a defined high fat diet (HF, 'Western fast food' diet from Speciality Feeds, Australia) and the other on normal diet (ND, Speciality Feeds, Australia) until six months of age. The HF diet contained 40% of total digestible energy derived from lipids, with 21% total fat, 4.7% crude fibre, 1000 IU/kg vitamin D3 and 0.6% calcium, whereas ND had 8.9% energy derived from lipids, with 4.8% total fat, 4.8% crude fibre, 2000 IU/kg vitamin D3 and 0.8% calcium.

Tumour model

BALB/c mice at age 6 months were implanted subcutaneously (s.c.) in one flank with $1x10^6$ CT26 tumour cells (undifferentiated, syngeneic colon adenocarcinoma from the American Type Culture Collection, Cryosite Distribution, Australia). Tumour volume was measured daily with callipers, and individual animals were sacrificed when tumours reached a maximum volume of 1000 mm^3 ($V = \pi/6 \times width^2 \times length$). The animals were sacrificed by isoflurane (Baxter, Deerfield, IL, USA) overdose and cervical dislocation, and the tumours excised, organs harvested and blood collected. Organs and tumours were weighed, and divided in half, with one half immediately flash frozen, and the remainder fixed using formalin. Serum, organs and tumours were stored frozen at -80^{o}C.

Immunohistochemistry

Formalin-fixed tumours were prepared for paraffin embedded (FFPE) blocks, sectioned at 3-5 μm and stained with Haematoxylin and Eosin (H&E) to show tissue morphology. Staining of phosphohistone H3 (pHH3) was used to determine tumour cell proliferation rates (Gerring *et al.* 2015). Immunostaining was carried out following standard protocols for heat acti-

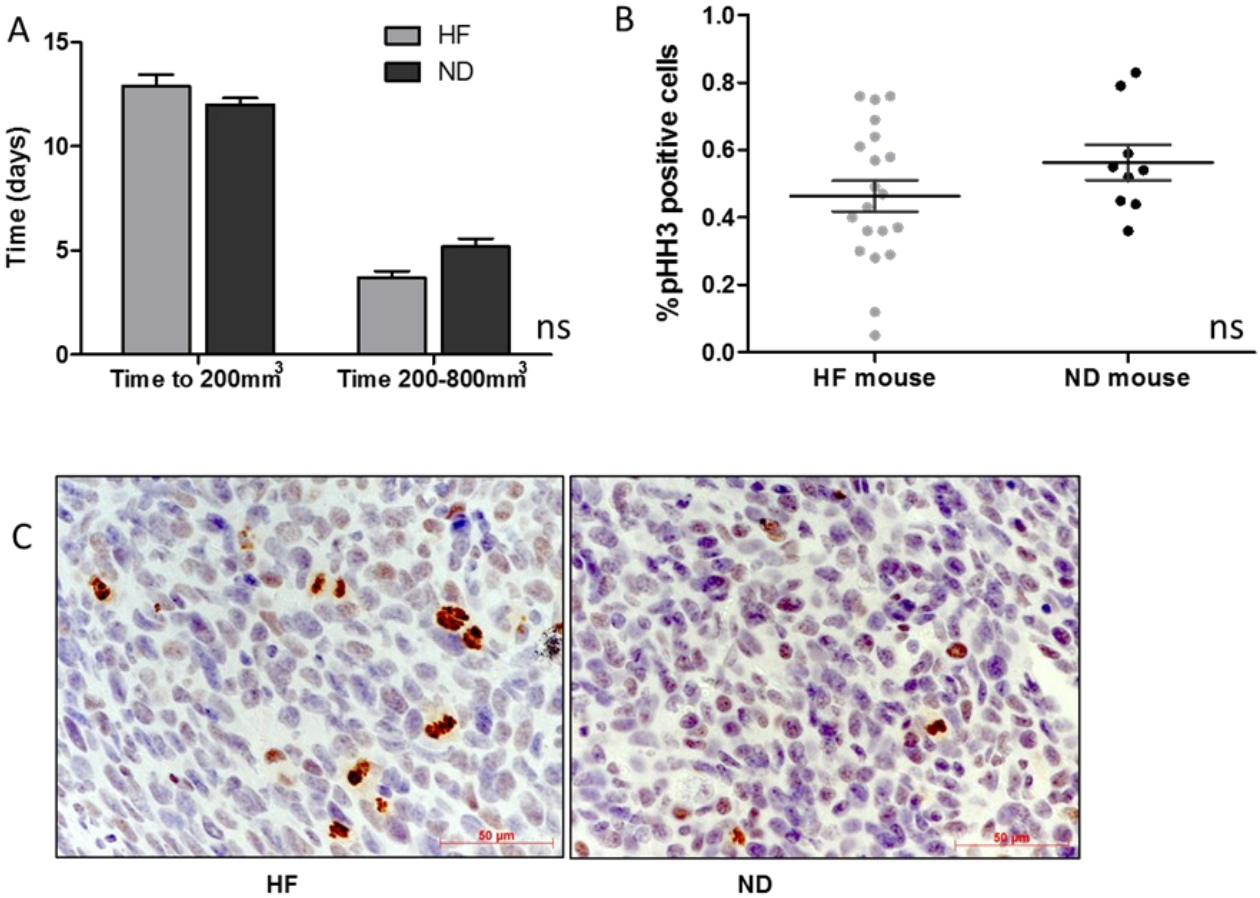


Figure 2. Tumour growth (A) and tumour cell proliferation (B, C) in mice fed western style, high fat diet (HF) or normal diet (ND). (A) BALB/c mice fed on HF or ND were implanted s.c. with CT26 tumour cells, and tumour growth was recorded as time taken to reach 200mm^3 and time to reach 4x tumour volume (200-800mm^3) (HF n=10, ND n=5). (B) Tumour sections were stained for the proliferation marker pHH3 and positive cells were counted in 12 random views, expressed as percentage of total tumour cells. (C) An example of pHH3 staining (brown) in tumour sections of mice fed HF or ND is shown; size bar indicated 50 µm. Data were analysed using 2-way ANOVA, ns = not significant.

vated antigen retrieval in citrate buffer to detect pHH3 (1:5000, Abcam, Melbourne, Australia) and using the Anti-mouse HRP-DAB Cell and Tissue staining kit (R&D Systems, Pharmaco, Auckland, NZ). Scoring of pHH3 was carried out on viable tumour tissue by counting positive cells in 12 random views at high magnification (x40 objective) for each section, relative to total number of cells per view.

Adipokine array

Serum and tumour lysates were used to identify changes in the adipokine profiles. Frozen tumour tissues were homogenised to a fine powder in liquid nitrogen using a chilled mortar and pestle. The tissue wet weight was measured and phosphate buffered saline with protease inhibitors (Sigma Aldrich, Auckland, New Zealand) was added to make a homogeneous suspension. Serum or lysates were pooled from n=4 mice per group (ND and 3 HF groups: HFF, HFM, HFS), and a ProteomeProfiler Mouse Adipokine Array (R&D Systems, Pharmaco, Auckland, NZ) was used to determine relative levels of selected adipokines according to manufacturer's instruction. The array allows the simultaneous detection of 38 different obesity-related molecules. Briefly, 40 µg of protein (tumour lysate) or 100 µl serum (1/100 dilution) were mixed with a cocktail of biotinylated antibodies, and identified with Streptavidin-HRP and chemi-luminescent detection. Relative protein levels were determined using pixel density (using Alliance 2.7 and UVI Band V12.14 software, Uvitec, Cambridge, UK) standardised to reference spots on the array.

Enzyme linked immune-sorbent assay (ELISA)

Insulin-like growth factor binding protein 5 (IGFBP-5) in serum and tumour lysates was quantified using an IGFBP-5 mouse ELISA kit (Abcam, Sapphire Bioscience, Redfern, Australia) according to manufacturer's instructions. Tumour lysate was used at 40 µg/100 ml and serum was diluted 1/100.

Statistical analysis

Statistics and graphical analysis were done using GraphPad Prism version 5.08. HF vs ND were compared using one and two way ANOVA, with post-hoc Bonferroni or post-hoc Tukey tests, as appropriate, with significance assumed as $p<0.05$.

Results

Male BALB/c mice are resistant to diet-induced obesity

After six months feeding on Western style, high fat (HF) diet, male BALB/c mice weighed significantly more (range 27 – 39g, $p=0.003$) than mice on standard normal diet (ND, range 27g – 34g), but this difference accounted for only about 10% of their weight (mean 33g vs 30g on day 158 for HF vs ND, respectively) (Figure 1A). This small difference can partly be accounted for by the observation that the mice preferred normal mouse chow to HF, as mice on HF consumed significantly less food ($p<0.001$, Figure 1B). Organ weights were similar in the two feeding groups, with the exception of omental fat, which weighed 2.7-times more in HF mice compared to ND mice ($p<0.001$, Figure 1C). As expected, tumour weights did not differ as they were removed when they reached a certain size (1000 mm^3), and not after a specified number of days post implantation.

Tumour growth rates and cell proliferation do not differ according to diet

Tumour growth rates, defined as lag phase (time from implantation to reach 200 mm^3) and log phase (time to reach 4x volume, i.e. growth from 200-800 mm^3) did not differ between the two feeding groups ($p>0.05$, Figure 2A). This was confirmed by staining for the pHH3 proliferation marker (Figure 2B and C), which showed no significant difference between tumours grown in mice on HF vs ND ($p>0.05$).

Resistance to diet-induced obesity affects tumour growth

Mice fed HF diet showed considerable variation in their final body weight as well as omental fat weight, and could be separated into three groups; those that became obese on HF diet (HFF), those that remained slim (HFS) and an intermediate group (HFM) (Figure 3A and B). When tumour growth was analysed accord-

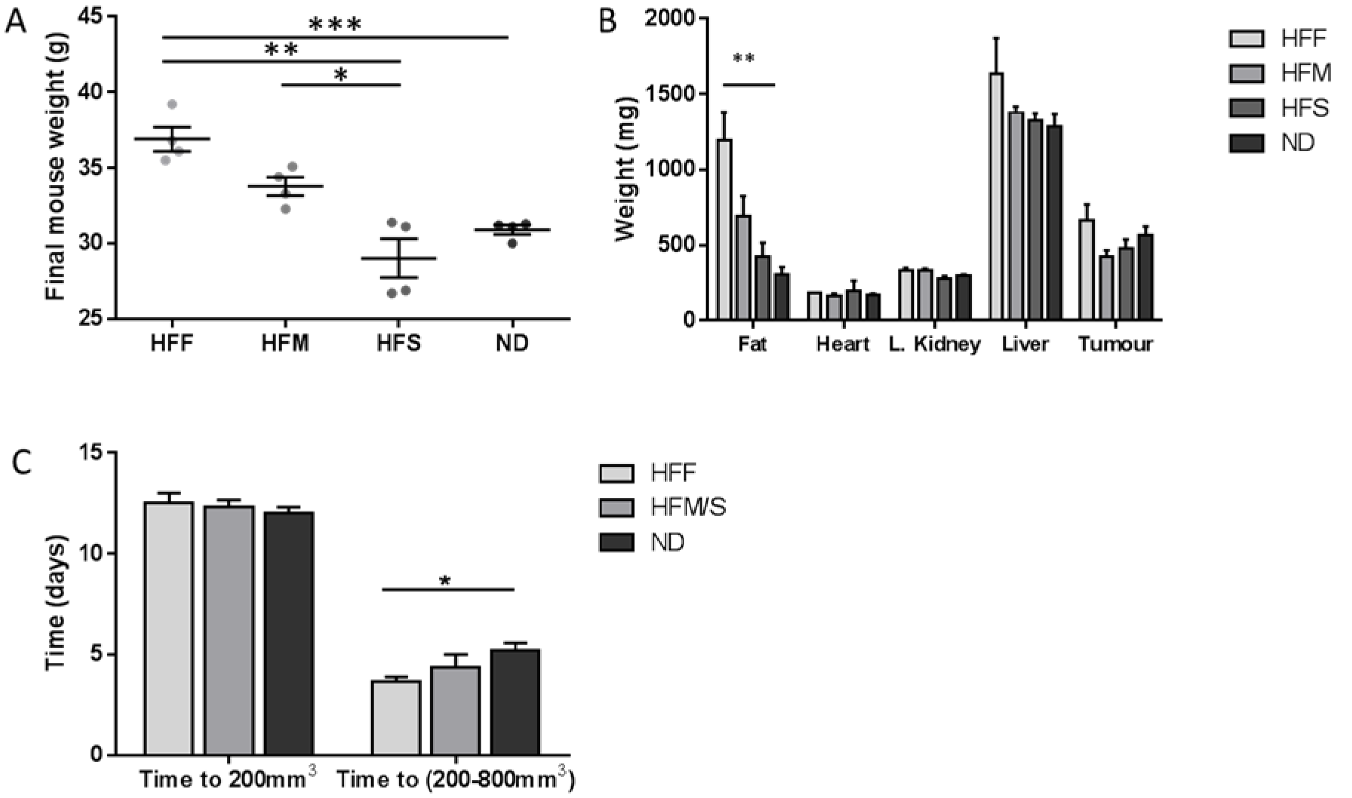


Figure 3. Subgroup analysis of mice more or less resistant to diet-induced obesity. BALB/c mice fed on HF diet were divided into those that became obese (HFF, n=4), those that stayed slim (HFS, n=4) and those in-between (HFM, n=4), and compared to mice on normal diet (ND, n=4). (A) Final mouse weight and (B) fat weights were significantly different in the subgroups. (C) Tumour growth was recorded as time taken to reach 200mm^3 and time to reach 4x tumour volume (200-800mm^3) (n=3-6). Data were analysed using 2-way ANOVA followed by post-hoc Bonferroni tests; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

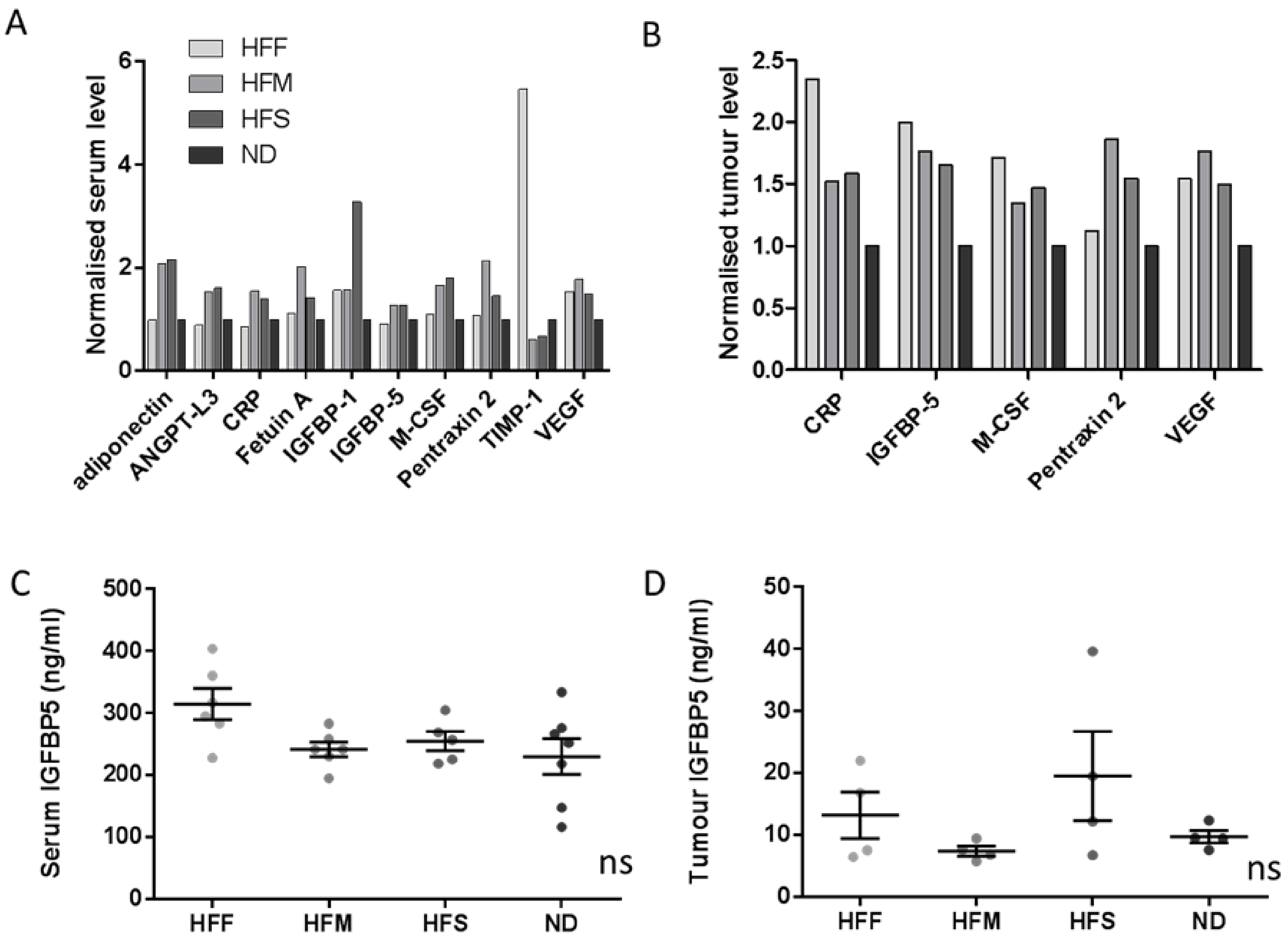


Figure 4. Protein array analysis and IGFBP-5 ELISA data of mice fed on Western style, high fat diet (HF) compared to normal diet (ND). BALB/c mice fed on HF diet were divided into those that became obese (HFF, n=4), those that stayed slim (HFS, n=4) and those in-between (HFM, n=4), and compared to mice on normal diet (ND, n=4). (A) Serum and (B) tumour lysates were analysed using an adipokine array, and IGFBP-5 concentrations were measured in (C) serum and (D) tumour lysates by ELISA. Data were analysed using 2-way ANOVA, ns = not significant.

ing to the mice's propensity to become obese, tumours in the HFF group grew significantly faster (200-800mm^3) than those in the ND group ($p<0.05$), and faster than the other two HF groups, although that was not significant ($p>0.05$) (Figure 3C). Staining for pHH3 showed that tumours in the HFS group had a lower proliferation rate than the other groups, but this did not reach significance ($p>0.05$, results not shown).

Resistance to diet-induced obesity affects adipokine profile

A range of 38 adipokines in the serum and tumour lysates of these groups of mice were analysed by proteomic profiling (Figure 4A and B, Supplementary table 1). In serum, of the 21 detected adipokines, 9 increased by more than 50% and none decreased by more than 50%, compared to serum from mice on ND (Figure 4A). A more than 2-fold increase was observed for adiponectin (HFM and HFS), fetuin A (HFM), IGFBP-1 (HFS), pentraxin 2 (HFM) and TIMP-1 (HFF), compared to ND in serum. Within the three high fat diet groups, all serum adipokines were increased in the two non-obese groups, compared to the obese group (with the exception of TIMP-1). In tumour lysates, of the 20 adipokines detected, 5 increased by more than 50% and none decreased by more than 50%, compared to lysates from mice on ND (Figure 4B). A more than 2-fold increase was observed for CRP (HFF) and IGFBP-5 (HFF) compared to ND in tumour.

IGFBP-5 was analysed further using ELISA (Figure 4C and D), as it showed differential levels in both serum and tumours. IGFBP-5 levels in serum were increased in HFF mice, whereas in tumour lysates the HFS group showed highest levels, compared to the ND group, but none were statistically significant ($p>0.05$, Figure 4C and D).

Discussion

This study confirmed that BALB/c mice are resistant to diet-induced obesity, but within this group of mice, some animals remained slim while others became obese. This inherent variance affected tumour growth such that obese mice on high fat diet showed increased tumour growth rates compared to mice on normal diet, whereas non-obese mice on high fat diet showed no difference in tumour growth rates. The implications of these findings is that high fat diet alone does not drive tumour growth, but rather inherent factors within the host.

Mouse models have been widely used to investigate the effect of diet-induced obesity on cancer risk and progression. Among several strains of laboratory mice, BALB/c mice were identified as one of the most resistant ones to diet-induced obesity (Fearnside *et al.* 2008, Montgomery *et al.* 2013, Nishikawa *et al.* 2012, Waller-Evans *et al.* 2013) although one study reported higher fat gain and hepatic lipid accumulation in BALB/c compared to others (Nishikawa *et al.* 2007). C57BL/6 mice are highly susceptible to diet-induced obesity and hence the majority of studies have been carried out in these animals. These C57BL/6 studies demonstrated that dietary factors contribute substantially to the risk of colorectal (Dougherty *et al.* 2009, Newmark *et al.* 2001, Richter *et al.* 1995, Riso *et al.* 1996) and other cancers (Xue *et al.* 1996). Yet tumour growth in our study relied primarily on the propensity of mice to become obese and not their diet *per se*.

This study did not show any effect of Western style, high fat diet *per se* on tumour growth. This is in contrast to two studies done by Park *et al.*, who demonstrated an increase in CT26 colorectal (Park *et al.* 2012) and 4T1 breast tumour (Kim *et al.* 2011) growth and metastases in BALB/c mice fed high fat diet. In a direct comparison between the study by Park *et al* (2012) and this study, the main differences were length of feeding (16 weeks vs 20 weeks), the method of tumour implantation (CT26 tumours were implanted in Matrigel vs PBS), and composition of the diet (60 kcal% fat vs 40% energy derived from fat, identical quantities of vitamins and minerals vs reduced vitamin D and calcium). Low calcium and reduced vitamin D were previously identified as important risk factors in the Western style diet in addition to high fat content (Erdelyi *et al.* 2009, Richter *et al.* 1995). Thus, these alterations may explain the differences in outcome between the studies, but also emphasise that enhancement of tumour growth by high fat diet may not be a universal fact, and that host factors are likely to play an important role.

In BALB/c mice, Western style diet has been shown to induce DNA binding activity of nuclear factor k-B and increase serum concentrations of tumour necrosis factor alpha (Kim *et al.* 2010), to change levels of choline-containing phospholipids (Kim *et al.* 2014), and to deregulate genes involved in fatty acid biosynthesis and uptake (Nishikawa *et al.* 2012), as well as to deregulate signalling pathways of the proteasome, PPAR signalling and ubiquitin-mediated proteolysis (Waller-Evans *et al.* 2013). Our data indicated that several adipokines changed with diet, with the majority showing increased levels in obesity-resistant mice compared to obese BALB/c mice. Exceptions were increased Tissue Inhibitors of Metallo-Proteinases (TIMP-1) levels in serum and increased C-Reactive Protein (CRP) levels in tumour lysates of HFF mice. TIMPs play a vital role in the development of obesity by supporting adipogenesis and extracellular matrix degradation (Crandall *et al.* 1997). *In vivo* studies have shown that elevated circulating levels of TIMP-1 derived from the primary tumour prepare the metastatic niche by upregulating factors in the liver that induce homing of tumour cells and promote formation of diffuse micrometastases in the liver tissue (Kopitz *et al.* 2007, Schelter *et al.* 2011). Our study observed no macroscopic metastases in the liver, but could not rule out single cell tumour dispersions. CRP is an acute-phase protein involved in inflammation and is associated with obesity (Nanri *et al.* 2007). Both TIMP-1 and CRP are likely to be of importance in tumour growth in obesity-resistant mice but further work is required to confirm our observations. The effect of diet and obesity on the IGF-1 axis in this study, specifically IGFBP-5, were subtle and similarly require further work. It is interesting that, although adiponectin was reduced in the HFF group of mice compared to HFS/M mice, as expected (Lamas *et al.* 2015), HFF mice had levels similar to the ND group.

In conclusion, tumour growth was enhanced in animals unable to resist obesity and this affected serum and tumour adipokine profiles. The implications of our data are that diet *per se* may not be the main driving factor in diet-induced obesity, but rather the host response to this challenge.

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