Evaluating invasive and non-invasive methods to determine fat content in the laboratory mouse

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Abstract: In the midst of an obesity epidemic in humans, diet induced obesity studies in rodents are fundamental to unravel the complex mechanisms underlying this disease, ultimately resulting in the identification of new preventative and therapeutic strategies. The current study was designed to determine if high throughput multi-object CT scanning was capable of providing precise quantification of adipose tissue in C57BL/6 mice when benchmarked to the gold standard method for evaluating fat mass (freeze drying). We report a strong correlation between body weight alone and fat percentage in our mouse cohort (20 g-40 g, r = 0.95). The gonadal fat depot was identified as the most accurate single predictor of total fat mass (r = 0.931). Importantly, we observed a high positive correlation between both live tissue weight and dissected adipose tissue when correlated to CT predictions (r ≥ 0.862), suggesting CT can accurately be used to predict total fat mass/percentage and non-fat mass/percentage in our cohort. We conclude that the use of multi-object *in vivo* CT fat quantification is cost effective, accurate and minimally invasive technique in the genetic manipulation era to exploit lean/obese genes in the study of diet induced obesity, allowing longitudinal studies to be completed in a high throughput manner.

Keywords: Adipose, C57BL/6, Computer Tomography, multi-object CT scanning

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1 Introduction

With the rapid advances in genetic approaches to whole organism physiology, utilising cell-specific gene deletions, the mouse has emerged as an unsurpassed model for the study of human diseases. The mouse model has many advantages compared to larger animal models, including; access to whole genome sequence data from multiple strains, short generation interval, high reproductive rate, low maintenance costs and the ability to control and standardise environmental factors [1,2]. Here we focus on the use of the mouse model to study body composition, which is essential in the midst of an obesity epidemic in humans.

Briefly, obesity is defined as abnormal or exaggeration of normal adiposity that may impair health resulting from a level of energy intake, which exceeds the body's energy expenditure (http://www.nhs.uk/conditions/Obesity/Pages/Introduction.aspx). Obesity plays a central role in the pathophysiology of diabetes mellitus, insulin resistance, dyslipidemia, hypertension, atherosclerosis constituting the metabolic syndrome [3]. Unlike other clinical manifestations, human obesity has no barriers. It prevails irrespective of gender, ethnicity, and age [http://www.who.int/mediacentre/factsheets/fs311/en/index.html, 4]. Alarmingly, over the past several decades, obesity has grown into a major global epidemic, currently the fifth leading risk of death worldwide, at a worldwide cost of approximately $147 billion annually [5-7]. However obesity is preventable. A huge global effort is currently effective to reduce obesity, through the promotion of healthy diet and increasing physical activity in the entire population. Coupled with this, scientific research is fundamental to unravel the complex and multiple forces driving the obesity epidemic resulting in the development and identification of new preventative, therapeutic and genetic strategies. Due to this complexity human studies can take decades; thus animal models, used in a synergistic way, are necessary to address the etiology, genetic and molecular aspects and the pathophysiology of obesity and evaluate potential treatments [8,9]. In brief,

many mouse models have been generated either utilising high fat diet or genetics to induce both obesity and its related pathologies. These models have recently been elegantly reviewed by Kanasaki & Koya [10]. In order to conduct repeated measurements on the same individuals (e.g. at different age points), the non-invasive and precise quantification of adipose tissue in the animal model is crucial.

Body weight coupled with dissection is the simplest and most common predictor of fat mass, however it has been previously reported that body weight alone is a poor predictor of fat percentage especially in leaner mice [11]. However, the dissection of single fat pads, exemplified by gonadal fat, has shown to be highly correlated to total body fat percentage but the experimental animals need to be sacrificed [12]. The gold standard methods for evaluating fat mass include freeze drying and chemical analyses. Briefly, freeze dried mass/wet weight allows for the prediction of fat percentage from dry matter content (FatP_FD) [13,14], subsequent chemical analysis of the dried and ground animal allows for the prediction of protein content (from nitrogen) [15]. These methods are time consuming and destructive implying they can only be used *post mortem* (with the exception of body weight) thus cannot be used in longitudinal studies.

In the last decade, many sophisticated modalities have emerged to quantify adiposity in both rodent and farm animal models in a non-invasive way, including both standard and small animal dual-energy X-ray absorptiometry (DXA, PIXImus DXA), *in-vivo* computed tomography (CT) and micro computed tomography (μCT), standard and micro magnetic resonance imaging (MRI, μMRI). Whilst these modalities have a high resolution, are quantifiable and allow for longitudinal studies, they have a low throughput and are relatively expensive (Table 1). They also require initial calibration against accepted gold

Table 1: Comparison of Invasive and Non-invasive methods for measuring adiposity in rodents.

	Dissection / Freeze Drying	**PIXImus DXA**	**μMRI**	**CT**	**μCT**
Brief Description	Individual fat pads removed from carcass and weighed. Whole carcass dried together with removed tissues.	Low energy X-rays to produce high-resolution (0.18 × 0.18 mm pixel) images.	Application of a strong magnetic field, combined with radio waves result in a detectable signal utilising the body's natural magnetic properties.	X-rays used to generate cross-sectional 2D models via rapid rotation of the X-ray tube 360° around the animal.	Synonymous to conventional CT, however produces very high resolution images allowing for "3d" microscopy.
Cost	Low	Medium	High	Medium	High
Image acquisition time (individual mice)	N/A	≈ 5 minutes	≈ 30 minutes	≈ 5 minutes (6 mice)	< 60 minutes
Manual / Automated analysis	Manual	Automated	Manual	Automated	Manual – Some automated functions.
Expertise /Software	Dissection skills, access to freeze dryer.	Software included	Require MRI Radiographer, free software available for analysis.	Radiographer, Software included.	Training required. Software included.
Outcomes	Isolated fat pads highly correlated to total fat mass. Dry matter gold standard predictor of fat %.	Estimated density and mass of lean, adipose, mineralised tissue.	Precise quantification of individual fat depots. Production of 2D/ 3D models. Further detailed analysis possible.	Estimated mass of lean, adipose and mineralised tissue. Production of 2D/3D models.	Precise quantification of individual fat depots. Production of 2D/3D models. Further detailed analysis possible.
Destructive	Yes	No	No	No	No (*in vivo* μCT)
References	[12-14]	[24, 25, http://piximus.com/]	[26-29]	[16, 30, 31]	[10, 32, http://www.bruker.com/products/x-ray-diffraction-and-elemental-analysis/x-ray-micro-ct.html]

standard methods such as dissection, freeze drying and chemical analysis [13,14].

The current study was designed to extend our preliminary data on ovine muscle using high throughput multi-object CT scanning to determine if this strategy was capable of providing precise quantification of adipose tissue of C57BL/6 mice, the most widely used of all inbred strains [16]. This approach offers the advantages of a quick, accurate and inexpensive method to assess adiposity in mice. Additionally we wished to determine if the simplistic weighing of subcutaneous (SB), gonadal (GF), mesenteric (MF) and interscapular brown (iBF) fat pads is indeed an accurate method to predict whole body adiposity.

2 Materials and Methods

2.1 Abbreviations

BW - Body Weight
CT - Computer Tomography
DM - Dry matter
DS - Dissected
DXA - Dual-energy X-ray absorptiometry
FatP - Fat percentage
FatW - Fat weight
FD - Freeze dried
GF - Gonadal Fat
HU - Hounsfield unit
iBF - Interscapular Brown Fat
LTW - live tissue weight
MF - Mesenteric Fat
MRI - Magnetic resonance imaging
SB - Subcutaneous Fat
STAR - Sheep Tomogram Analysis Routines
TW - Total Weight

Mice – All animal experiments were approved by The Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals. 20 male C57BL/6 inbred mice of varying body mass (20-40 g) and age (35 to 200 d) were sacrificed by cervical dislocation and immediately weighed.

CT scanning – The bodies of freshly sacrificed mice were immediately CT-scanned using a Siemens Somatom Esprit Computer Tomography (CT) Scanner. Multi-object (6 mice in one scan), cross-sectional CT images were taken along the length of the body (3 mm apart, field of view 450 mm, approximately 70 images per mouse) (Fig.1) [17]. Sheep Tomogram Analysis Routines (STAR) software (BioSS - V.4.8; STAR: Sheep Tomogram Analysis Routines, University of Edinburgh, http://www.ed.ac.uk) was used to calculate the total area and average densities of fat, muscle and bone in each carcass image without gutting (segmenting out guts and organs), based on density thresholds (low fat: -174 HU, high fat: -12 HU, low muscle: -10 HU, high muscle: 92 HU, bone: < 94 HU). These values were established from sheep calibration trials in which lambs underwent CT scanning followed by slaughter and full dissection [18-20]. Mouse specific thresholds were not available and have not been reported in the literature to the best of our knowledge.

Dissection – Following CT scanning, individual fat pads (SB, GF, MF & iBF) were extracted and weighed (Figure 1B and C). Whole mouse carcasses were subsequently frozen at -20 °C prior to freeze drying.

Freeze drying – Whole mouse carcasses and corresponding isolated adipose tissue were freeze dried to determine the dry matter weight (DM) of the carcass. The predication of individual fat percentage values was calculated by regression on dry matter content (DM/BW) using an equation (FatP _DM/BW (%) × 113 - 30.2) derived by Hastings & Hill [13]. The CT based measures of tissue weights were then compared to the DM-based estimates for the fat content (fat %) and the fat free mass (FFM) in (%) using simple linear regression: $y_i = b_0 + b_1 x_i$, with b_1= regression coefficient and b_0 = intercept.

Statistical data analysis – The data analysis has used linear regression and correlation analysis based on Excel (Microsoft Office 10) built-in functions with interval of confidence and testing of the correlation coefficients according to standard procedures described in the statistical literature [21, 22]. Data are presented as means ± standard error (SEM) were appropriate. Regression and correlation coefficient's are given with the intervals of confidence (P = 0.05).

3 Results

3.1 Liveweight, fat and non-fat traits measured by freeze drying, CT and dissection

The description of the dataset regarding these traits in terms of simple means and their standard errors is given in Table 2. The LWT of the mice was on average 31.5 g, but splitting into the age groups shows a high variation between the age group means (20.9 g to 41.1 g). This produced the required variation in the fat traits, with fatness increasing with age. Taking the FatP_FD as an

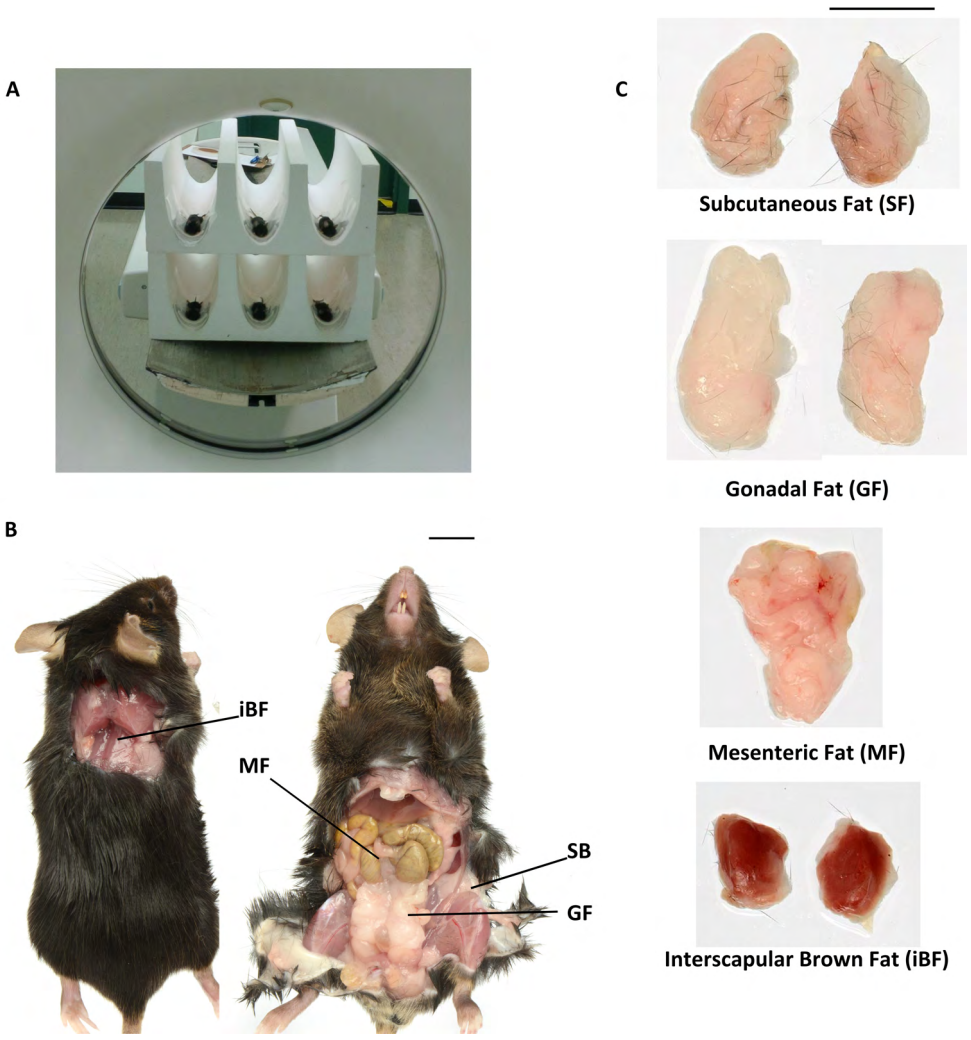

Figure 1: A. Multi-object CT scanning, **B.** Photograph depicting locations of fat depots (iBF). Scale bar = 1 cm, **C.** Representative Images of the dissected fat depots. From top to bottom: Subcutaneous Fat (SB), gonadal fat (GF), mesenteric fat (MF), interscapular brown fat (iBF). Scale bar = 1 cm.

example, the fat content increases from about zero % (-1.1%) at 35 days to 18% at 200 days (with an average of 9%). Given the different methods to measure the size of the fat and non-fat compartment of the body it is not unexpected to find that the magnitude of the measured quantities of fat and non-fat differed between the methods. The total estimated fat by FD across all ages amounts to 3.4 g (9.0%). The value obtained from CT is 6.5 g (23.6%) and thus much higher, probably indicating that the thresholds derived from sheep dissection trials need to be refined for mice. Another opportunity is to use the obtained CT values in suitable regression equations to predict accurately the fat values obtained by freeze drying, the gold standard. Similarly, it is not unexpected to find the lowest total fat amount from dissecting out the 4 above mentioned fat depots (SB, GF, MF and iBF). This method finds on average 1.5 g of the total fat (sum of the 4 depots), which is less than the half of the existing body fat. Again, the total body fat can be easily predicted from appropriate regression equations which either use the information from one or all dissected fat depots, as will be shown further below.

3.2 Liveweight as a predictor for fat and non-fat

The simplest predictor of fatness is often live weight (Table 3). This assumes however that there is a wide variation in fatness as in our cohort, resulting from the use of mice from 35 days to 200 days of age. Both, FatW_FD and FatP_FD are highly correlated with LWT, r = 0.95 and 0.95, respectively, indicating that LWT alone allows good prediction of the fat weight and content in this sample of mice. LWT also correlates highly with the non-fat weight

Table 2: Simple means for liveweight, fat and non-fat traits measured by freezer drying, CT and dissection with their standard errors of the mean (SEM); n = 5 per age group).

	35 days		120 days		180 days		200 days		All Ages	
Trait	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
LWT (g)	20.88	0.373	30.18	0.946	33.88	1.130	41.12	0.900	31.525	1.970
FatW_FD (g)	-0.244	0.172	2.251	0.478	3.963	0.893	7.576	0.468	3.387	0.681
FatP_FD (%)	-1.141	0.818	7.400	1.426	11.416	2.264	18.401	0.946	9.019	1.994
NFatW_FD (g)	20.76	0.430	27.65	0.848	29.35	0.495	32.47	0.610	27.56	3.237
NFatW_FD (%)	100.78	0.412	92.34	1.391	88.05	2.115	80.62	0.778	90.45	5.636
FatW_CT (g)	2.640	0.120	4.87	0.618	7.07	1.181	11.34	0.698	6.48	0.709
FatP_CT (%)	16.06	1.030	19.86	2.030	51.77	1.560	32.48	1.850	23.58	1.660
NFatW_CT (g)	13.91	0.577	19.39	0.221	19.90	0.881	23.57	0.841	19.19	2.692
NFatW_CT (%)	83.94	1.032	80.14	2.033	74.08	3.708	67.52	1.853	76.42	2.033
TW_CT (g)	16.55	0.520	24.25	0.656	26.97	0.980	34.91	0.742	25.67	1.729
M_DS (g)	0.050	0.006	0.151	0.013	0.194	0.022	0.468	0.053	0.216	0.031
GF_DS (g)	0.143	0.006	0.508	0.057	0.784	0.176	1.680	0.120	0.779	0.116
SB_DS (g)	0.126	0.007	0.237	0.031	0.383	0.057	0.645	0.108	0.348	0.041
iBF_DS (g)	0.096	0.011	0.156	0.007	0.179	0.023	0.257	0.019	0.172	0.017
NFatW_DS(g)	20.47	0.367	29.13	0.889	32.34	0.876	38.07	0.767	30.00	4.749
NFatW_DS (%)	98.02	0.068	96.53	0.235	95.53	0.608	92.60	0.557	95.67	1.565
TW_DS (g)	0.414	0.016	1.052	0.092	1.540	0.258	3.050	0.270	1.514	0.201

Note: Animals of different ages have been chosen to enlarge the variability in the fat traits.

and content estimated by FD: r = 0.98 and 0.95. It is of note that these seemingly good prediction abilities of LWT will be diminished when looking at mice at one age or at animals with a small age span only, although the low sample sizes per age group in our study do not allow us to prove this. As the FD measures of fatness are highly correlated with those obtained from CT it is not surprising to find LWT also a good predictor for the CT based traits, with the correlations slightly lower (r = 0.85 to 0.94; Table 3) .

3.3 Benchmarking CT predictions against freeze drying

Water content is a robust indicator of fat proportion as described previously and can be easily measured by freeze drying [12]. This method is cheaper and quicker than the equivalent chemical analysis. As the latter was not available in our study, freeze drying was the chosen benchmarking method. The initial use of both methods on the same sample allows the derivation of prediction equations, which can be utilised to allow the use of subsequent CT measures alone to predict fat and non-fat traits given the results of both methods correlate highly. Here we show high positive correlations between CT measured fat (FatW_CT and FatP_CT) and the corresponding traits quantified by FD (r = 0.91 to 0.98). Moreover, we also observed high positive and just slightly lower correlations between the non-fat traits measured by FD and CT. It seems as the correlations between the measures expressed as a percentage are always slightly lower than the correlations between absolute values.

3.4 Benchmarking Isolated dissected fat pad mass against freeze drying and CT

The dissection of a single isolated fat pad from mice is a very common, invasive but highly simplistic and rapid exercise to evaluate total fat mass in mice. However the accuracy of this in C57BL/6 mice has not yet been reported. High positive correlations (r = 0.92, 0.93, 0.98 and 0.89, respectively) were found between all isolated fad pads and the FatW_FD (g) with the highest correlation between GF_DS and FatW_FD indicating that the gonadal fat pad seems the best single trait predictor for the total body fat in a mouse (r = 0.98; Table 3). Again, as the FD and CT measured fat traits are highly correlated it is expected that the mass of the individual fat depots correlates also

Table 3: Correlation coefficients and confidence intervals for CT and dissected fat predictions (n = 20).

	Correlation Coefficients	95 % Confidence Interval (lower bound)	95 % Confidence Interval (upper bound)	Regression Coefficients (b_1)	5% Lower Limit	5% Upper Limit	Intercept (b_0)
LTW (g) vs. FatW_FD (g)	(b0)	0.876	0.980	0.387	0.325	0.450	-8.824
LTW (g) vs. FatP_FD (%)	0.947	0.869	0.979	0.967	0.806	1.127	-21.445
LTW (g) vs. NFatW_FD (g)	0.979	0.947	0.992	0.613	0.550	0.675	8.824
LTW (g) vs. NFatP_FD (%)	0.947	0.979	0.869	0.967	0.806	1.127	121.445
LTW (g) vs. FatW_CT (g)	0.936	0.842	0.975	0.443	0.361	0.525	-7.745
LTW (g) vs. FatP_CT (%)	0.850	0.654	0.939	0.886	0.616	1.156	-4.438
LTW (g) vs. NFatW_CT (g)	0.894	0.747	0.958	0.442	0.333	0.551	5.258
LTW (g) vs. NFatP_CT (%)	0.850	0.939	0.654	0.886	0.616	1.156	104.348
FatW_CT (g) vs. FatW_FD (g)	0.983	0.957	0.994	0.848	0.771	0.925	-2.105
FatP_CT (%) vs. FatP_FD (%)	0.905	0.770	0.962	0.886	0.681	1.091	-11.867
NFatW_CT (g) vs. NFatW_FD (g)	0.915	0.794	0.966	1.158	0.907	1.408	20.804
NFatP_CT (%) vs. NFatP_FD (%)	0.905	0.770	0.962	0.886	0.681	1.091	111.867
TW_ CT (g) vs. TW_FD (g)	0.987	0.966	0.995	1.100	1.012	1.189	-5.978
SB_DS (g) vs. FatW_FD (g)	0.918	0.800	0.967	12.117	9.54	14.694	-0.829
M_DS (g) vs. FatW_FD (g)	0.931	0.830	0.973	17.202	13.873	20.531	-0.322
GF_DS (g) vs. FatW_FD (g)	0.982	0.954	0.993	4.931	4.463	5.400	-0.454
iBF_DS (g) vs. FatW_FD (g)	0.891	0.741	0.957	41.115	30.839	51.391	-3.682
SB_DS (g) vs. FatW_CT (g)	0.938	0.846	0.975	14.358	11.74	16.976	1.483
M_DS (g) vs. FatW_CT (g)	0.932	0.832	0.973	19.98	16.151	23.808	2.171
GF_DS (g) vs. FatW_CT (g)	0.981	0.952	0.993	5.716	5.163	6.269	2.027
iBF_DS (g) vs. FatW_CT (g)	0.862	0.677	0.944	46.092	32.736	59.447	-1.445
TW_DS (g) vs. FatW_FD (g)	0.979	0.947	0.992	2.865	2.574	3.157	-0.952
TW_DS (g) vs. FatP_FD (%)	0.938	0.848	0.976	6.870	5.625	8.115	-1.383
TW_DS (g) vs. FatW_CT (g)	0.982	0.953	0.993	3.332	3.013	3.650	1.434
TW_DS (g) vs. FatP_CT (%)	0.908	0.777	0.963	6.787	5.244	8.329	13.303

highly with the CT based fat mass measures, with the highest value (r = 0.98) between the GF_DS and FatW_CT emphasising the good prediction opportunities if only one depot is being used, and highlighting the accuracy of multiple-object CT.

The last 4 rows of Table 3 highlight that the prediction accuracy can be increased if the fat weight found in all 4 depots is summed up and all correlated to FD and CT measured fat mass. The correlations are both 0.98 and therefore quite similar to the GF_DS vs. FatW ones.

4 Discussion

Previous publications have evaluated the use of body weight alone as predictor of fat mass or fat percentage, reporting a good correlation in obese mice, yet a poor correlation in very lean mice [11]. Contrary to this, we report a strong correlation between body weight alone and fat mass and percentage in mice of 20 g (35 days of age) to 40 g (200 days of age) measured by freeze drying and by multi-object CT. As a tendency the r values were slightly lower for the relative measures (%) compared to

the absolute values (g), and the correlation to FD measures seem slightly higher compared to the CT based measures. These high correlations are likely due to the large age span present in our cohort, selected to produce a large variation in fat traits. However in more similar body weights this may not be the case and the use of LWT as solely predictor of fat mass is not recommended.

Compared to the LWT as a fat predictor, the multi-object CT yielded slightly higher accuracies, e.g. the FatW_CT is a very good predictor for the total fat mass in the body (r = 0.98). The chosen approach in our study to CT scan freshly killed mice allows conclusions for CT scanning mice *in vivo*. The excellent prediction abilities of multi-object CT allow the implementation of experimental designs which are without it impossible to realise. For example one could feed mice with a "normal" diet over a certain time period, then CT scan these mice and change to a high-fat or high calorie diet and CT scan the same mice again. The results would be very informative for studies into the problem of diet induced obesity. Our study shows the fat amount in live animals can be predicted very well with multi-object spiral CT. There was only limited research exploring the use of multi-object CT in fat mass prediction in mice. This study now provides prediction equations based on one predictor (Table 3), indicating that CT can accurately predict the degree of adiposity in the murine model. Moreover, with the use of multiple regression analysis a further small increase in accuracy could be expected, however we wanted to use simple predicators at this stage. It also may be possible to improve accuracy by optimising the thresholds for the mouse model and segmenting out the guts in the process of CT-image analysis.

As this study has highlighted, simplistic dissection is also sufficient to quantify whole body adiposity, and the measurement of one depot (gonadal fat pad) achieves the same predictions accuracy than the dissection of all 4 depots. These results agree with previous work [23] and are based on the strong positive correlation between isolated fat depots and predicted fat mass.

More complex questions with regard to adiposity, such as the study of fat distribution in mice, muscle shape and hepatic fat/water ratios will require more sophisticated, high resolution imaging techniques such as μMRI or μCT, which are inaccessible to many and are accompanied by high costs, increased analysis image and acquisition time. We conclude that the use of multi-object *in vivo* CT fat quantification is a highly valuable, cost effective, accurate and minimally invasive technique in the genetic manipulation era to exploit lean/obese genes in the study of diet induced obesity, without the sacrifice of the animal, allowing longitudinal studies to be completed in a high throughput manner.

4.1 Future work

The importance of mouse models in scientific research is indisputable. In order to abide by the principles of *3 R's, a*dopting new technology or optimising current technology to meet changing needs is fundamental. In addition to the parameter's we measured, multi-object CT with appropriate benchmarking, will have the capability to accurately predict total muscle and bone mass (as shown in other species), thus replacing time consuming dissection in experimental design. Additionally further work is required to benchmark both μMRI and μCT to dry matter based prediction or chemical analyses. These modalities, unlike CT provide high spatial resolution and contrast, allowing not only quantification of adiposity in longitudinal studies, but also the ability to distinguish between normal and pathological tissues. However for simple adiposity measurements, allowing for the dissection of the genetic basis of diet induced obesity and study of diet effects over age, we believe that CT is currently unsurpassed.

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