Supplement 1

Model and curve fit for fat cell data measuring average fat cell volume against body fat mass

Summary

Three data sets relating fat mass to the average fat cell volume were analyzed and fitted to a simple quantitative model. The analysis strongly suggests that, over a wide range of fat mass (from leanness to extreme obesity), increasing fat mass is a result of enlargement of fat cells plus increased number of fat cells.

Theory

The central assumption we make is that the number of fat cells, denoted n, has a lower limit n_0, which could be the number of fat cells from birth, and may also increase linearly with the total fat mass m (unit: kg) in the body with a proportionality constant k_f (unit: kg^{-1}), i.e.

$$n = n_0 + k_f \cdot m \qquad (1)$$

Note that a very low k_f corresponds to the notion that fat cells hardly increase in number in adult humans.

We now take interest in the average volume per cell, and note that the volume V_{tot} of the total body fat can be written $V_{tot} = g_f^{-1} \cdot m$, where g_f is the density of body fat ($0.9 \cdot 10^{-12}$ kg/pl)[1]. The average volume per cell, V, is written $V = V_{tot} / n$ and insertion of equation 1 gives

$$V = \frac{V_{tot}}{n} = \frac{g_f^{-1} \cdot m}{n_0 + k_f \cdot m} = \frac{\left(g_f \cdot n_0\right)^{-1} \cdot m}{1 + n_0^{-1} \cdot k_f \cdot m} \qquad (2)$$

This equation describes a saturation curve for the average volume per cell with respect to the total body fat mass. Important to note is that when k_f equals zero, we have:

$$V = \left(g_f \cdot n_0\right)^{-1} \cdot m \qquad (3)$$

i.e. the average volume per cell increases linearly with the total body fat mass if there is no increase in the fat cell number.

For the cases where we have the BMI of the subjects instead of bioimpedance based estimations of m, we need to estimate m from the BMI value in order to use equation (2). For this, we use a previously described formula based on age, BMI and sex[2]. We can now fit equation (2) to m versus average fat cell volume, the parameters estimated from the data fit will be $a = \left(g_f \cdot n_0\right)^{-1}$ and $b = n_0^{-1} \cdot k_f$. Since g_f is known *a priori*, we can from a and b obtain estimates of n_0 and k_f, and the model is hence fully identifiable.

An issue that needs to be addressed is the variability of the observable parameters (a and b) between individuals. Generally we have, for subject i, $y_i = \left(a_i x_i\right)/\left(1 + b_i x_i\right) + e_i$, where $y = V$, $x = m$, and e is the error in measurement, assumed to be independent from x. The model is over-parameterized if a and b are allowed to vary freely between individual subjects – a perfect fit can be obtained by letting $a_i = y_i/x_i$ and $b_i = 0$. In the following, we outline how to assess the individual variability of a_i and b_i. Let us introduce the error model $a_i = a + c_i$ and $b_i = b + d_i$. A Taylor expansion gives

$$y_i = \frac{(a+c_i)x_i}{1+(b+d_i)x_i} + e_i \approx \frac{ax_i}{1+bx_i} + \frac{c_i x_i}{1+bx_i} - \frac{d_i a x_i^2}{(1+bx_i)^2} + e_i, \qquad (4)$$

where the first term is the original model and the following three are errors. These errors can be assessed by observing the residuals $\left| y_i - \frac{\hat{a}x_i}{1+\hat{b}x_i} \right|$, where $\hat{a}$ and $\hat{b}$ are estimated from a curve fit of equation (2) using a least squares method. If the residuals vary little with respect to $x_i/(1 + bx_i)$ and $ax_i^2/(1+bx_i)^2$, respectively, individual variations in a and b are negligible; if they grow with any of these terms there is significant variation in a and/or b, respectively.

Given a low individual variability in the parameters, a likelihood ratio test to compare the model to the null hypothesis, the special case that k_f is 0 (whereby fat cells do not form in the adult human), can be performed accordingly: let $z_1 = \sum_i \left(y_i - \frac{\hat{a}x_i}{1+\hat{b}x_i} \right)^2$, i.e. the residual sum of squares. Similarly, $z_2 = \sum_i (y_i - \hat{a}x_i)^2$, corresponding to the independent fit of the null hypothesis that $k_f = 0$, and thereby $b = 0$. If p is the number of observations, the null hypothesis is rejected if

$$p \times \log \frac{z_2}{z_1} > \chi_\alpha^2(1), \qquad (5)$$

where $\chi_\alpha^2(1)$ is the α-quantile of a χ^2 distribution with one degree of freedom, and α is the chosen significance level. The reason for having one degree of freedom is that the null hypothesis has one less parameter than the full model. As a complement to the likelihood ratio test, we also calculated Akaike's information criterion (AIC)[3] for the two models, equation (2) and (3). A model is by this criterion favoured over another one by a lower AIC score.

Data Fit and Statistical Tests

We partitioned our data into four data sets: one consisting of samples of subcutaneous fat (women), one consisting of samples of subcutaneous fat (men), one consisting of subcutaneous fat (men and women) where total body fat mass was measured using bioimpedance (a subset of the two former groups) and one consisting of samples of visceral fat (men and women). The subjects are described in Supplement 4. From each subject, we have the total body fat mass, as directly measured or as estimated from BMI, and the average cell volume. Curve fits of equation (2) to these three data sets were performed in the least squares sense using Matlab's statistical toolbox. The individual values are shown if Fig.1. The estimated parameter values together with their 95% confidence intervals (obtained using standard Gaussian large sample theory) are given in Table 1.

As described in the theory section, we checked whether the fit of equation (2) was significantly better than a fit to the linear equation (3). Regressions were made of the residuals against $x/(1+bx)$ and $ax^2/(1-bx)^2$ respectively. As presented in table 2, the coefficient of determination r^2 was always smaller than 0.05, which justified the straightforward use of the likelihood ratio test (equation 5).

In all cases, a likelihood ratio test showed that k_f is significantly greater than zero ($P < 0.001$). Furthermore, in all cases, Akaike's information criterion favoured equation (2). Thus the data strongly suggest that the fat cell number and the fat cell size increase with the BMI at all levels of body weight in adult subjects.

Data set	Method for obtaining m	$(g_f \cdot n_0)^{-1}$	$n_0^{-1} \cdot k_f$
Subcutaneous fat (women n=480)	Calculated from BMI	42 ± 5	0.033 ± 0.006
Subcutaneous fat (men n=190)	Calculated from BMI	53 ± 7	0.046 ± 0.010
Subcutaneous fat (men and women n=474)	Bioimpedance	39 ± 4	0.029 ± 0.005
Visceral fat (men and women n=135)	Calculated from BMI	27 ± 8	0.025 ± 0.012

Table 1. Estimated parameters. Intervals are 95% confidence intervals.

Data set	r^2 for R_1	r^2 for R_2	LR score	AIC score, equation (3)	AIC score, equation (2)
Subcutaneous fat (women n=480)	8.6×10^{-4}	8.4×10^{-4}	481 ($P < 0.001$)	5306	4827
Subcutaneous fat (men n=190)	0.023	0.031	259 ($P < 0.001$)	2114	1857
Subcutaneous fat (men and women n=474)	2.8×10^{-4}	6.5×10^{-4}	435 ($P < 0.001$)	5170	4737
Visceral fat (men and women n=135)	0.010	0.0090	62.0 ($P < 0.001$)	1476	1416

Table 2. Coefficients of determination r^2 for the residual plots against $R_1 = x/(1 + bx)$ and $R_2 = ax^2/(1 - bx)^2$ of the three data sets, outcome of the likelihood ratio (LR) test (equation 5), and AIC score for fits to the linear equation (3) and the nonlinear equation (2), respectively.

References Supplement 1

1) Fidanza, F, Keys A, and Anderson JT. Density of body fat in man and other mammals. *J Appl Physiol* **6**:252-256 (1953).

2) Gallagher, D., et al. How useful is body mass index for comparison of body fatness across age, sex, and ethnic groups? *Am J Epidemiol* **143**:228-239 (1996).

3) Akaike, H. A new look at the statistical model identification. *IEEE Trans Automat Contrl*

19:716-723 (1974).

Supplement 2

Table showing all data from the 35 people whose adipose DNA was carbon dated. DOB = date of birth to the year. Weight reported is the weight in connection with removal of adipose tissue. BMI values given are the pre-bariatric surgery values. For a complete explanation of this, and the terms 'C14' and 'pMC', refer to Supplement 4

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Table number	Fat sample number	Sex	DOB	Age (yrs)	BMI (kg/ m^2)	Height (cm)	Body weight (kg)	Fat cell volume (pl)	Fat cell weight (mg)	Total number of fat cells in the body (10^{10})	^{14}C	+/ -	pMC	+/ -	Yr of analysis
1	16	F	1984	21	23.1	164	62	497	455	3.527	74.000000	29.000000	1.08130000	0.02910000	2006.3
2	22	M	1982	23	21.1	171	62	417	382	1.889	157.539498	12.351154	1.16540720	0.01235115	2005.9
3	41	F	1978	27	22.8	162	60	597	546	2.880	107.100000	7.900000	1.11450000	0.00790000	2005.9
4	43	F	1976	29	24.2	176	75	505	482	4.446	121.798572	7.492636	1.12942334	0.00749264	2005.9
5	15	F	1976	29	29.0	164	78	442	405	6.889	86.000000	6.500000	1.09330000	0.00650000	2005.6
6	53	F	1976	29	29.1	174	88	249	228	13.864	69.000000	6.000000	1.07630000	0.00600000	2006.3
7	60	M	1977	28	38.6	170	80	419	383	5.498	90.000000	31.300000	1.09750000	0.03150000	2007.0
8	34	F	1975	30	32.8	171	96	452	414	9.646	134.625190	13.168813	1.14233714	0.01316881	2005.9
9	29	F	1975	30	24.2	160	62	576	527	3.376	143.747461	5.426034	1.15152142	0.00542603	2005.9
10	51	F	1974	31	35.6	165	97	733	671	6.640	79.900000	11.500000	1.08730000	0.01150000	2006.3
11	18	F	1974	31	20.5	174	62	465	425	3.396	80.000000	11.000000	1.08730000	0.01100000	2006.3
12	20	F	1973	32	24.0	168	68	492	450	4.331	179.600000	5.900000	1.18790000	0.00590000	2005.6
13	12	F	1973	32	23.5	170	68	457	418	4.540	100.000000	5.300000	1.10734204	0.00531092	2005.6
14	40	F	1973	32	39.0	165	70	488	446	4.898	160.565841	17.803280	1.16845411	0.01780328	2005.9
15	38	F	1973	32	31.2	163	83	668	611	5.360	94.000000	30.000000	1.10140000	0.03020000	2006.3
16	7	F	1971	34	38.6	168	85	486	445	7.272	86.200000	11.700000	1.09320000	0.01170000	2005.2
17	8	F	1970	35	40.2	160	62	190	174	10.458	149.700000	4.700000	1.15770000	0.00470000	2005.2
18	47	F	1970	35	46.7	168	98	471	431	14.348	91.000000	68.000000	1.09840000	0.06840000	2006.3
19	4	F	1970	35	21.2	161	55	462	422	3.239	133.000000	9.600000	1.14060000	0.00960000	2005.7
20	36	F	1969	36	28.2	163	75	542	496	5.365	150.325058	10.831066	1.15814372	0.01083107	2005.9
21	11	F	1968	37	37.4	173	83	466	408	7.092	121.700000	7.000000	1.12923651	0.00697875	2005.2
22	14	F	1968	37	22.1	162	58	461	422	3.637	124.000000	4.800000	1.13151255	0.00478965	2005.6
23	42	F	1968	37	22.6	176	70	472	432	4.409	186.493591	9.581095	1.19455809	0.00958109	2005.9
24	21	M	1968	37	26.6	187	93	707	646	3.125	171.000000	10.000000	1.17890000	0.01000000	2005.7
25	48	M	1966	39	28.1	174	85	492	450	4.575	198.638748	5.853327	1.20678579	0.00585333	2005.9
26	27	F	1955	50	59.6	169	107	654	599	15.076	115.379213	7.858549	1.12296035	0.00785855	2005.9
27	5	F	1954	51	54.1	166	103	414	378	20.750	173.000000	15.000000	1.18103860	0.015488162	2005.7
28	13	F	1954	51	35.4	163	65	479	438	4.731	144.200000	6.900000	1.15181483	0.006925132	2005.6
29	50	F	1949	56	23.9	153	56	540	494	3.586	142.454642	6.666165	1.15021981	0.006666165	2005.9
30	3	F	1948	57	35.9	160	72	535	489	5.604	149.500000	4.700000	1.15719760	0.004715856	2005.6
31	25	F	1945	60	26.1	167	73	548	502	5.155	103.100000	5.400000	1.11047968	0.005392452	2005.6
32	46	F	1944	61	41.0	166	80	550	504	6.338	179.328271	9.393897	1.18734407	0.00939390	2005.9
33	45	M	1935	70	24.4	173	73	521	477	5.234	133.000000	8.900000	1.14084402	0.00885455	2007.0
34	19	F	1934	71	29.4	168	83	718	657	5.284	129.749413	18.914663	1.13760000	0.01900000	2005.9
35	35	F	1933	72	27.5	165	75	521	477	6.145	75.000000	17.100000	1.08240000	0.01720000	2007.0

Supplement 3

Modelling of ^{14}C data

Summary

We used a mathematical model for birth and death of adipocytes to estimate adipocyte turnover in 35 adult subjects. Death and birth rates of adipocytes were calculated from DNA ^{14}C content in adipocytes, ages of patients, and absolute adipocyte number. We used three scenarios for the evolution of adipocyte formation at early ages and estimated rates in three BMI categories: lean (BMI<25 kg/m^2), overweight (25 kg/m^2≤BMI<30 kg/m^2) and obese (BMI≥30 kg/m^2) subjects. We conclude that the adipocyte death rates in the three BMI groups are not different (Kruskal-Wallis test, p=0.6). Absolute production of new adipocytes is significantly higher in obese subjects than in lean subjects (Kruskal-Wallis test, p<0.01). The adipocyte relative turnover number, however, does not differ significantly between BMI groups (p-value>0.1): a value of 8.4 ± 6.2% adipocytes (median ± absolute deviation) is replaced each year. Using two other data sets, we determined the age of onset of adipocyte number expansion, and the relative growth in cell number during that expansion.

Model

We used a linear model with an age-structure for birth and death of adipocytes to estimate kinetic parameters of adipocyte birth and death. Data come from three sources: subjects who underwent cosmetic surgery and subsequent analysis of ^{14}C in subcutaneous fat cell DNA (D1), cross-sectional data from a study by Knittle et al[1] (D2) and from subjects analysed for the relationship between BMI and subcutaneous fat cell volume (D3). The subjects in D1 and D3 are described in Supplement 4. Available from D1 data are average ^{14}C levels in

adipocytes samples collected during liposuction, ages of subjects, the absolute body adipocyte count, and their BMI (n=35). From D2 data, we have the average adipocyte number for different age groups, for normal or obese children (ages range from 0.5 y to 18 y). D3 data consist of ages, BMI, and adipocyte numbers (n=687) using lean (BMI<25 kg/m^2), and obese (BMI≥30 kg/m^2) subjects. We sorted D1 patients in three BMI categories: lean (BMI<25 kg/m^2), overweight (25≤BMI<30 kg/m^2), and obese (BMI≥30 kg/m^2) patients. The ages of subjects ranged from 21 to 72 years (mean ± STD, 39.9 ± 13.8 y, at date of collection) and the BMI values from 20.5 to 59.6 kg/m^2 (mean ± STD, 30.4 ± 9.1 kg/m^2). ^{14}C levels varied from 1.0763 to 1.2068 percent Modern Carbon (mean ± STD, 1.134 ± 0.036 pMC) (Table 1).

Table 1 Summary of subjects' data D1

n=35	Birth year	Age (y)	^{14}C levels (pMC)	Adipocyte number (x10^{10})	BMI (kg/m^2)
Mean	1965.09	39.91	1.13435	6.36	30.39
STD	13.86	13.81	0.03617	3.98	9.15
Median	1970.00	35.00	1.13760	5.23	27.70
1st quart	1954.50	30.50	1.09990	3.98	23.95
3rd quart	1974.50	50.00	1.15792	6.76	35.50
Min Value	1933.00	21.00	1.07630	1.89	20.50
Max Value	1984.00	72.00	1.20679	20.75	59.60
Lean n=13	1969.62	35.38	1.13711	3.73	22.89
Overweight n=8	1958.38	46.63	1.13049	6.30	28.00
Obese n=14	1964.71	40.29	1.13399	8.84	40.44

The age-structured model for cell birth and death has four parameters: the cell death rate γ, the initial age t_0 at which the adipocyte distribution is known (we refer to the age of the patient with t and the age of adipocytes with a), the initial cell number N_0, and the production of new adipocytes from a fat stem cell pool β. We make the following assumptions.

Period of expansion of adipocyte number: Most of the adipocytes in a young adult are born during a specific, critical period (starting at t_0) before adulthood. This means that in a subject aged t_0 years, all adipocytes have age 0 years (or perhaps very few older cells from childhood).

Constant cell death rate: All adipocytes, regardless of their age, die at a rate γ that is constant over the lifetime of the person.

Constant cell production: Adipocytes are produced steadily at a rate β that is independent of the total cell number and is constant over the lifetime (after age t_0). This is because mature adipocytes do not divide and we ignore regulation of new adipocytes by mature cells.

Mathematically, these assumptions may be formulated as a linear partial differential equation with an age-structure[2]:

$$\frac{\partial n(t,a)}{\partial t} + \frac{\partial n(t,a)}{\partial a} = -\gamma n(t,a) \qquad (1)$$

The function n(t,a) is the density of adipocyte of age a for a subject of age t (unit of n: cells/year, units of a and t: year). The initial condition at $t = t_0$ is $n(t_0,a) = N_0\ \delta_0(a)$, where δ is the Dirac delta function (i.e. all cells at t_0 have age $a = 0$) and N_0 is the initial adipocyte number (unit: 10^{10} cells). A boundary condition describes the birth of new adipocytes from progenitor cells, $n(t,0) = \beta$ (unit: 10^{10} cells/year). Equation 1, with the initial and boundary conditions, is related to the more general McKendrick-von Foerster equation used in population dynamics[2]. This equation may be solved cells with an age smaller than the time elapsed since t_0. For $0 \le a \le t - t_0$,

$$n(t,a) = N_0 \delta_{t-t_0}(a) e^{-\gamma a} + \beta e^{-\gamma a} \qquad (2)$$

and the average age of a adipocyte in an adult of age $t > t_0$ is

$$\langle a \rangle = \frac{\gamma(t-t_0)e_t - b(t-t_0)e_t + b/\gamma(1-e_t)}{\gamma e_t + b(1-e_t)} \qquad (3)$$

where $e_t = e^{-\gamma(t-t_0)}$. The newly introduced parameter b is the birth rate relative to the initial cell number N_0,

$$b = \frac{\beta}{N_0}.$$

(The parameter b is useful since $\langle a \rangle$ becomes independent of the total cell number). The total cell number at age t, $N(t)$, is found by integrating $n(t,a)$ over the ages of adipocytes a,

$$N(t) = \int_0^{t-t_0} n(t,a)da = \frac{\beta}{\gamma}(1-e^{-\gamma(t-t_0)}) + N_0 e^{-\gamma(t-t_0)}. \qquad (4)$$

The equation satisfies the initial condition $N(t_0) = N_0$. If $b > \gamma$, the cell number increases with time, else decreases. The cell number always stabilises to a constant steady state $N^* = \beta/\gamma$. To relate the age of the adipocytes with the ^{14}C data, we used a carbon normalization curve K (ref. 3). If the adipocyte age distribution follows Equation 2, the average ^{14}C level C is

$$C = \frac{\int_0^{t-t_0} K(a)n(t,a)da}{N(t)}. \qquad (5)$$

Equation 5 depends on four parameters: N_0, γ, β, and t_0. The parameter N_0 can be eliminated by dividing the numerator and denominator by N_0, and by replacing the term β/N_0 with b as in Equation 3. Thus three parameters remain to be estimated: γ, b, and t_0. In general, it is not possible from Equation 5 alone to estimate all three parameters; different choices of γ, b, and t_0 can yield the same value of C. This is why additional assumptions on one or more parameters must be made.

Default Scenario: The death rate γ, and the ages of the patients are sufficiently large to neglect the initial adipocyte population, i.e. N_0 is very small. This means that $b = \beta/N_0$

becomes large and t_0 can be set to $t_0 = 0$. Parameter b cancels out in Equation 5, and the model has only one parameter left to estimate: the death rate γ. This allows us to calculate an individual death rate for each patient. The adipocyte production can then be estimated from the adipocyte count of each subject. Under Default Scenario, $n(t,a) = \beta\, e^{-\gamma a}$, for $a \leq t$, and 0 otherwise.

By comparing the results with those obtained with different assumptions, we may be able to identify information that is statistically significant. In addition to the Default Scenario, we used the following alternative scenarios:

Scenario A: Same as Default Scenario, except that an average death rate for each group is calculated instead of individual values for each subject. This scenario allows comparing parameter estimates obtained with the assumptions from the Default Scenario and Scenario B and C, in which average values are also estimated. Parameter estimates are obtained for the different BMI groups.

Scenario B: The cell turnover as we model it starts in a patient at age t_0, and for age $t \geq t_0$ the total cell number is constant, i.e. $b = \beta/N_0 = \gamma$. Thus γ and t_0 need to be fitted. Parameter estimates are obtained for the different BMI groups.

Scenario C: The cell turnover as we model it starts in a patient at age t_0, which has to be guessed. Parameters γ and b are fitted. Parameter estimates are obtained for the different BMI groups.

As shown below, the model is robust with respect to t_0 (changing t_0 does not affect the estimates of other parameters), as long as this parameter is far from the ages of patients at time of collection. The consequences of each case are felt on the cell numbers. In the Default Scenario and Scenario A, the population increases to reach a steady state. In Scenario B, the

population is constant, by definition, for $t \geq t_0$. In Scenario C, the population start at an initial value and then can either increase or decrease before reaching the steady state, depending on the fitted values of γ and b.

Statistical methods

Where the data were normally distributed, we used the mean ± standard deviation (STD) as a measure of central tendency and dispersion. Comparisons of means between groups were made using analysis of variance (ANOVA), which is a test used to compare the means of normally distributed groups having the same variance. Where data were not normally distributed, as assessed by the Jarque-Bera test for normality, we used the median ± absolute deviation (AD) as a measure of central tendency and dispersion. The absolute deviation is defined as the mean absolute difference of the data to the median. The absolute deviation is to the median what the standard deviation is to the mean is the sense that the median and the mean minimize their respective deviations. Comparisons between non-normally distributed groups were made using the Kruskal-Wallis test for equality of medians, which is a non-parametric ANOVA test and does not assume the data to have any specific distribution. We defined two groups to be statistically different if the p-value is less than 0.01. We also confirmed the results obtained with the Kruskal-Wallis test with a t-test (p-values not shown).

Results

Ages of subjects and BMI distribution

Subjects were divided according to their BMI and age. Data D1 are summarized in Table 1. Overweight patients tend to be older than lean and obese patients, but not significantly (ANOVA test, $p>0.05$). Similarly, we found no differences in ^{14}C levels between young and old patients and between lean and obese subjects (ANOVA test, $p=0.4$).

Estimating birth and death rates

Table 2. Summary of the adipocyte kinetic rates for three groups of subjects

	Default Scenario		Scenario A		Scenario B			Scenario C			
BMI category	γ mean±STD (median±AD)	β mean±STD (median±AD)	γ fit [CI]	β mean (median)	γ fit [CI]	β mean (median)	t_0 fit	γ fit [CI]	β mean (median)	b fit	t_0 g.
lean (n=13)	0.097±0.080 (0.082±0.053)	0.39±0.26* (0.32±0.18)	0.075 [0.028]	0.31 (0.29)	0.079 [0.047]	0.30* (0.28)	6.7	0.081 [0.050]	0.30* (0.29)	0.065	9
overweight (n=8)	0.15±0.12 (0.12±0.09)	1.1±1.5 (0.62±0.94)	0.093 [0.053]	0.61 (0.50)	0.091 [0.049]	0.57 (0.49)	5.7	0.11 [0.08]	0.57 (0.54)	0.027	9
obese (n=14)	0.11±0.06 (0.095±0.051)	1.1±0.6* (0.79±0.47)	0.088 [0.023]	0.81 (0.64)	0.079 [0.019]	0.70* (0.54)	19.7	0.075 [0.023]	0.75* (0.61)	large	9
all (n=35)	0.11±0.08 (0.084±0.062)	0.83±0.93 (0.50±0.53)	0.085 [0.016]	0.57 (0.45)	0.088 [0.015]	0.55 (0.46)	10.7	0.089 [0.025]	0.57 (0.47)	0.088	9

Lean (BMI<25 kg/m^2), overweight (25≤BMI<30 kg/m^2), and obese (BMI≥30 kg/m^2). Units are γ y^{-1}, β 10^{10} cells/y, b ICP/y; and t_0 y. [CI] 95% confidence interval on the parameters, STD standard deviation, AD absolute deviation, g. guessed. *Kruskal-Wallis test p<0.01.

Table 3 Age of adipocytes, mean years ± STD, (median years ± AD)

BMI category	Default Scenario	Scenario A	Scenario B	Scenario C
lean (n=13)	9.9±3.5 (10.2±2.7)	10.2±1.4 (10.1±1.0)	8.2±2.2 (8.5±0.8)	9.7±1.1 (9.9±0.9)
overweight (n=8)	8.8±4.8 (8.7±4.1)	9.8±0.9 (9.7±0.7)	9.7±1.6 (9.7±0.4)	10.5±0.8 (10.5±0.6)
obese (n=14)	9.7±4.0 (9.8±3.3)	9.9±0.7 (9.7±0.6)	9.4±1.3 (9.2±1.6)	10.3±0.7 (10.2±1.3)
all (n=35)	9.5±3.9 (10.2±3.3)	10.0±1.0 (9.9±0.8)	9.0±1.8 (9.2±0.8)	10.2±0.9 (10.2±0.7)

Since it is not possible to estimate all three parameters at once from equation for C, we used the Default Scenarios and the three scenarios described above. For each of them, the adipocyte death rate, γ, can be estimated. Moreover, in Default Scenario, γ is the only parameter, allowing estimates for individual patients unlike the alternative scenarios (in Scenario A, we calculated a single average death rate using the same assumptions as in Default Scenario). In Scenario B, t_0 is estimated together with γ, while $b = \gamma$ by definition.

Finally in Scenario C, γ and b are estimated together, while t_0 is assigned a guessed, fixed value. For each scenario, we calculated the death rate γ for the lean, overweight, obese, and all subjects. We estimated the death rates using the nonlinear fitting algorithm *nlinfit* from Matlab (www.mathworks.com).

Once the death rate estimated, we used the measured number of adipocytes in each patient (Supplement 4 contains the procedure for adipocyte number calculation) and their age to calculate the absolute birth rate β. This was performed by taking the total number of adipocytes given by the model ($N(t)$ in Equation 4) and solving for β. This leads to three formulas.

Default Scenario and Scenario A, $\beta = \gamma \frac{CELLNUMBER}{1 - e^{-\gamma AGE}}$. (6)

Scenario B, $\beta = \gamma CELLNUMBER$. (7)

Scenario C, $\beta = \gamma b \frac{CELLNUMBER}{b - (b - \gamma)e^{-\gamma(AGE - t_0)}}$. (8)

The equations are the direct consequence of the assumptions on each scenario. Results are displayed in Table 2. The average age of adipocytes $\langle \alpha \rangle$ was directly calculated from γ, b, and t_0 using Equation 3 (Table 3). *CELLNUMBER* is the calculated total number of fat cells in the body (details in Supplement 4), and *AGE* is the age in years of the subject.

Analysis

The death rates calculated from various scenarios are very similar, with values just below 0.09 per year (Table 2). This is encouraging, because estimating the absolute birth rates requires robust estimates of the death rates, cf. Equations 6, 7, and 8. No significant difference in death rates could be found between the lean and obese subjects (Kruskal-Wallis test for equality of medians, p=0.6). No significant differences in ages of adipocytes were found between different BMI categories (adipocyte age mean ± STD, 9.9 ± 3.5, 8.8 ± 4.8 and 9.7 ± 4.0 years for lean, overweight and obese subjects respectively, Table 3), which is a consequence of the ^{14}C levels being constant between BMI groups. A constant death rate across BMI categories should correspond to a higher adipocyte production in patients with high BMI. Four estimates were obtained for the birth rate β. Estimates varied between 0.29×10^{10}cells/y in lean subjects to 1.1×10^{10}cells/y in obese patients. For Default Scenario, the difference in β between lean and obese subjects is significant (medians 0.32×10^{10}cells/y and 0.79×10^{10}cells/y, Kruskal-Wallis test, p=0.003, Table 2). When divided into two groups with BMI below and above 27 kg/m^2, the difference is also significant (Kruskal-Wallis test, p=0.01). The difference in β between lean and overweight, and obese subjects, however, is not significant (p=0.7).

To test whether the results were robust with respect to the choice of t_0 =0 y in Default Scenario, we estimated the death rates with t_0 =5 and 10 y. No significant difference was found between death rates estimated with t_0 =0 y, and t_0 =5 y or t_0 =10 y (Kruskal-Wallis test, p>0.15), although the later onset of expansion (t_0 =10 y) was compensated by a decrease of 6% of the median death rate. We also estimated the death rates using different values of t_0 for lean (t_0 =10 y) and obese (t_0 =2 y) subjects. The difference of death rates was then close to the significance level (Kruskal-Wallis test, p=0.07).

It is possible that the turnover is not uniform among the adipocytes sampled. The ^{14}C labelling method measures the ages of cells present in samples and, by extension, their death rates.

Strawford et al.[4] used heavy water (^2H_2O) to label newly synthesised DNA with deuterium and reported extremely high turnover rates of 58%–105% per year, in subcutaneous adipose tissue aspirates in humans. Differences in tissue samples cannot account for the observed difference in rates, thus the difference in turnover rates suggests that the newborn adipocytes, i.e. differentiated post-mitotic pre-adipocytes, have higher turnover than the mature adipocytes. Including fast proliferating cells present in a proportion reported; 10% to 17% newly divided cells after 9 weeks of labelling[3], would affect our estimates by less than 10%. The actual 7–12 fold difference in turnover rates might represent the excess ratio of pre-adipocyte to adipocyte produced. These turnover rates indicate that most proliferating pre-adipocytes do not permanently join the adipocyte population. Nuclear bomb test ^{14}C and heavy water labelling are complementary approaches suitable to detect cell turnover in the older, mature adipocyte fraction and to estimate the faster turnover of the proliferating fraction.

In summary, the ^{14}C samples allowed us to estimate reliably two parameters of the model: the annual death rate γ and the annual adipocyte production β. We only obtained poor estimates for the two remaining parameter, t_0 and b, because of the finite memory of the adipocyte population. These parameters depend on childhood and cannot be estimated in adults. To get an estimate for these two parameters, we used cross-sectional data coming from children and adults.

Estimating t_0 and b from cell number data

Since t_0 and b cannot reliably be estimated from the ^{14}C sample, we used cross-sectional data on cell numbers in subjects with wide range of age (data set D2 and D3, Table 4) to see how the modelled cell number $N(t)$ varies over age. The aggregated data were divided into two groups, normal and obese (normal, BMI<25 kg/m^2; obese, BMI>30 kg/m^2), corresponding to the division used by Knittle et al[1].

Table 4 shows a summary of the data, divided according to age and BMI. Cell number does not change significantly between age groups in data set D3 (multi-group ANOVA, p=0.68). No data are provided for obese aged less than 3 since no obese were detected at this early age. We thus used data from the normal group for obese below 3 years old. We fitted the four parameters of the cell number $N(t)$ to these data and got estimates of t_0 = 5.7 ± 0.8 y and 2.1 ± 0.9 y and b = 1.3 ± 0.3 IC/y and 2.4 ± 0.6 IC/y for normal and obese respectively (Figure 1). Using the fitted adipocyte number curves, we calculated the age at which 75% or 90% of adult cell number is reached in lean and obese subjects. The adult adipocyte cell number was reached at 16.5 ± 1.3 y (90%) and 10.5 ± 1.3 y (75%) in the obese and at 18.5 ± 0.7 y, (90%) and 14.5 ± 0.7 y, (75%) in the lean (fitted values ± 95% confidence intervals on the predicted ages). The value of t_0 is consistent with the conclusions of Knittle et al.[1] that cell number expansion starts earlier in obese than in lean children.

	normal			Obese		
age	n	cell number	STD	n	cell number	STD
0.5	*18*	*0.4*	*1.4*	-	-	-
1	*27*	*0.5*	*1.5*	-	-	-
3	*31*	*0.9*	*1.2*	*17*	*1.9*	*1.2*
6	*29*	*1.1*	*3.1*	*23*	*3.4*	*3.3*
9	*29*	*1.2*	*3.2*	*15*	*4.0*	*3.1*
12	*22*	*2.8*	*2.6*	*27*	*6.2*	*5.1*
15	*16*	*3.5*	*3.0*	*29*	*6.8*	*4.3*
18	*6*	*4.0*	*2.0*	*9*	*8.0*	*3.0*
18	**6**	**4.4**	**2.1**	-	-	-
22	**21**	**4.1**	**2.1**	**20**	**8.7**	**3.4**
27	**42**	**4.6**	**1.7**	**49**	**7.6**	**2.0**
32	**44**	**5.2**	**2.3**	**55**	**7.3**	**2.4**
37	**24**	**5.0**	**1.5**	**56**	**7.3**	**2.3**
42	**56**	**4.2**	**1.8**	**59**	**7.3**	**2.1**
47	**27**	**4.3**	**1.6**	**47**	**7.7**	**2.4**
52	**16**	**4.4**	**2.4**	**22**	**7.5**	**2.7**
57	**9**	**4.9**	**2.1**	**21**	**7.1**	**2.3**
60+	**8**	**4.2**	**0.9**	**13**	**6.0**	**2.4**

Table 4. Adipocyte number for different age groups and BMI categories. Data in italics are D2[1], and in bold D3 (present study).

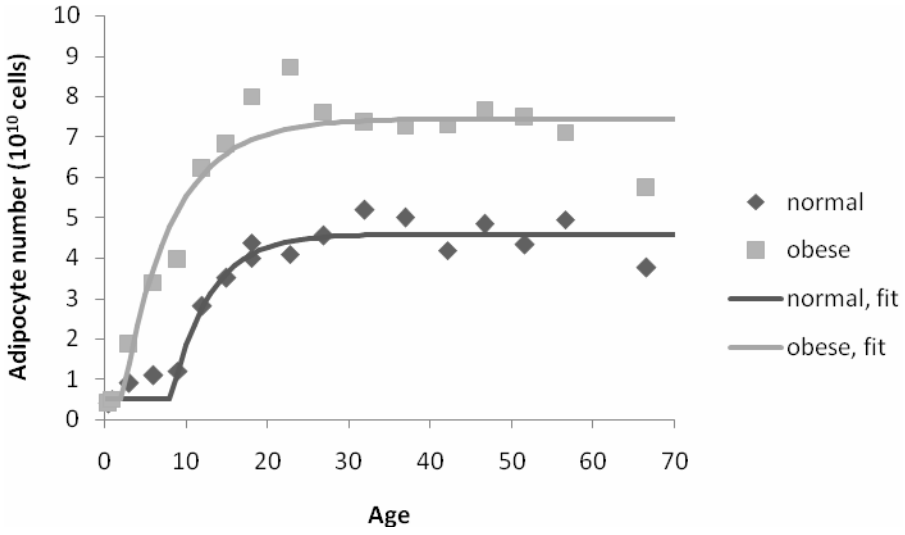

Figure 1. Results of the fitting of the cell number data by $N(t)$

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Supplement 4

Subjects

The relationship between total body fat mass and fat cell volume was determined on two study groups. Abdominal subcutaneous adipose was obtained from 480 women and 190 men (age 16-72 years, mean 42 years) by biopsy under local anesthesia. For all subjects BMI and fat cell volume were calculated. A BMI based formula (see below) was used to determine fat mass, and men and women were analysed separately. On a subgroup of 357 women and 117 men we made direct measurement of fat mass with bioimpedance using GuadScan 4000 (Bodystat ltd, Isle of Man, British Isles). These men and women were grouped together for analysis so as to increase statistical power and because no gender differences in the relationship between fat mass and fat cell volume were found. Visceral (omental) adipose tissue was obtained at the beginning of elective abdominal surgery because of a non-malignant disorder from 84 women and 51 men (age 16-62 years, mean 39 years). In this group we had only information of BMI and fat cell volume and fat mass was determined using the BMI based formula. The effect of weight reduction was studied in 9 men and 11 women (age 26-57 years, mean 43 years) who were operated for obesity using gastric banding. An abdominal subcutaneous fat biopsy was obtained as above, before operation when BMI was 42.2 ± 4.5 (mean ± SD) kg/m^2 and 2 years thereafter when BMI was 34.6 ± 6.0 kg/m^2. In this group fat mass was determined with bioimpedance. For ^{14}C analysis, a large piece (about 200 g) of subcutaneous adipose tissue was obtained from 35 separate individuals (age 21 - 72 years). This group included 25 women and 4 men who underwent abdominal subcutaneous liposuction for cosmetic reasons, and 6 women who underwent abdominal wall reconstructive surgery 1-2 years after bariatric surgery for obesity, when weight reduction had caused skin folding of the abdomen. According to self-report the

"cosmetic surgery group" had not altered their adult body weight in an important way as long as they could remember and the bariatric surgery subjects had been overweight or obese since childhood. The subjects who had been operated with bariatric surgery were classified as obese regardless of their BMI at the time of reconstructive surgery, since their surgical diagnosis was massive obesity and we can show in this study that weight reduction does not influence fat cell number. In subjects participating in ^{14}C analysis fat mass was determined by the BMI based formula. The studies were explained to each subject and his/hers consent was obtained. Ethical permission for all studies was approved by the ethics committee at Karolinska Institute.

Isolation of adipocytes from adipose tissue

Fat cells were isolated from the remaining components of the tissue according to the collagenase procedure of Rodbell[1]. In brief, tissue was cut into ≈ 20 mg pieces and incubated (1 g tissue/ml medium) in Krebs-Ringer phosphate (KRP buffer (pH 7,4) containing 4% bovine serum albumin (BSA) and 0.5 mg/ml of collagenase type 1 for 60 min at 37^{o}C in a shaking water bath. The isolated fat cells were collected on a nylon mesh filter and were washed 4-5 times with 0.1% KRP-BSA buffer. The purity of the isolation procedure was estimated by investigating 200 cells under a light microscope in each subject. The number of isolated cells not resembling fat cells or cell material that was stuck to a fat cell was always 0-2 per 200 counted cells.

In order to exclude possible contamination with even small numbers of non-fat cells in the preparations of mature fat cells, a number of techniques were used. Results are presented in Table 1. As a first step, 10^6 isolated mature fat cells were centrifuged at 200 g for 10 min. The resulting pellet, predominantly containing contaminating non-fat cells, was very small and hardly discernable by visual inspection and was termed the contaminating fraction. The

pellet was resuspended in a small amount of minimal essential medium (with no phenol red) and stained with 0.3 mg/ml of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) for 1 hr at 37C. Cells were lysed with isopropanol and staining was determined spectrophotometrically at 592 nm as described previously[2]. The amount of living cells in these preparations was compared with known amounts of isolated preadipocytes (see above) isolated from the same specimen and resuspended in different test tubes. With this semiquantitative titration comparison, the total number of surviving cells in the "contaminant" fraction was low and corresponded to 13-16 $x10^4$, i.e. a contamination degree of approximately 1.3-1.6%. In order to determine which type of cells the contaminant fraction was composed of, we performed flow cytometry. For this, contaminant fractions were obtained from approximately $10x10^6$ cells. These cells were washed and re-suspended in phosphate buffered saline (PBS) and incubated on ice for 30 minutes with conjugated, monoclonal antibodies against CD34 (preadipocyte marker), CD45 (common leukocyte marker), CD31 (endothelial cell marker) (all antibodies from Becton Dickinson, San Jose, CA, USA). Nonspecific fluorescence was determined, using equal aliquots of cell preparation incubated with anti-mouse monoclonal antibodies (Becton Dickinson, San José, CA, USA). Finally, the cells were assayed on a FACS Calibur flow cytometer (Beckton Dickinson, San José, CA, USA) and using Cellquest software (Becton Dickinson, San José, CA, USA). This analysis revealed that the contaminating fraction consisted of approximately 5-6 x 10^4 cells out of which 67-87% were living cells. The percentage of contaminating cells in total would be less than 0.5% if estimated in this manner. This was in accordance, albeit somewhat less, than the numbers estimated with the MTT assay. When comparing the cell numbers with the total amount of mature fat cell from which the contaminant fractions were obtained, the contamination degree for cells positive for each epitope was the following; CD34-0.041%; CD31-0.11% and for CD45-0.017%. The number of putative leukocytes present in the

contaminant fraction was finally assessed by incubation with CD45-Dynabeads (Dynal Biotech, AS, Oslo, Norway). In brief, 10^5 mature fat cells were resuspended in PBS+0.1% BSA. 1X107 Dynabeads were washed in PBS+0.1% BSA and added to eppendorf vials. After letting the tubes stand in a magnetic rack for 1 minute, the supernatant was removed and the mature fat cells were added to a final volume of 1 ml. Cells were allowed to incubate in the presence of Dynabeads for 20 minutes at +4C after which the tubes were put in magnetic racks. The supernatant was removed and the pellet, containing rosetted leukocytes was resuspended in Dulbecco´s modified Eagles medium (DMEM/F12) and plated on to cell culture plates. After allowing the cells to sink for an hour at 37C the number of rosetted leukocytes was counted. With this method the number of leukocytes in the contaminating fraction was estimated to <0.1% Finally, the methods employed above indicate that a small proportion of cells, probably 1% or less are present in washed and packed fat cells. However, it is still possible that a small number of cells may attach to large fat cells and thereby still be present in the mature fat cell fraction. In order to exclude this possibility, washed preparations of mature fat cells were stained with the nuclear stain 4'6-Diamino-2-phenglindole (DAPI) from (SIGMA, St Louise, MO, USA). In brief, mature adipocyte fractions were fixed in cell fix (BD Biosciences) for 20 min, followed by washes in PBS. DAPI (stock dissolved in methanol) was added at 100 ng/ml final concentration for 20 min followed by repeated washes in PBS. Cells were then mounted on microglass slides. Inspection by fluorescent microscopy in 10 random fields revealed clearly discernable nuclei in mature fat cells but no nuclei corresponding to contaminating cells on the surface of any fat cell or in between the adipocytes, clearly demonstrating that the mature fat cell preparations are more or less exclusively composed of mature fat cells with a very low degree of contaminating cells.

Method	Contamination degree (mean %)	Range (%)	*N*
MTT	1.45	1.3-1.6	2
FACS			3
CD34	0.041	0.0067- 0.07-	
CD31	0.11	0.011-0.2	
CD45	0.017	0.0061- 0.027	
Dynalbeads	<0.1		2

Table 1. Assessment of contamination of non-fat cells in the isolated fat cell preparation, using a variety of techniques, as described in the text. N = number of experiments.

Measurement of fat cell weight, volume, and number

Mean fat cell volume and weight were determined on isolated fat cells as follows: Fat cell diameter was determined during direct microscopy and the mean diameter of 100 cells in each individual was determined. The mean fat cell volume and weight were calculated by formulas developed by Hirsch and Gallian[3]. Coefficient of variation for the method is 2-3%. The mean values are essentially the same as those determined from fat cell sizing of intact pieces of human adipose tissue[4]. Total number of fat cells in the body was calculated as the amount of body fat divided by the mean fat cell weight. It is well known that mean fat cell volume and weight differs between various adipose regions in man. However, the differences are small and only introduce a marginal error when just one depot is used for the calculation of total fat cell number as discussed[5]. In the whole cohort total fat cell number (mean$\pm$SD) was 5.6$\pm$2.6 x 10^{10}, which is in the same range as in several other studies[5]. When BMI instead of bioimpedance was used to determine body fat we utilized an established formula based on age, BMI and sex to determine percentage of body fat[6]. Total body weight times percentage body fat gives the total fat mass. In a separate methodological study we evaluated this formula by comparing percentage body fat obtained by the formula and by dual-X-ray absorbmetry (DEXA) in 7 men and 14 women with BMI 17-46 kg/m^2 and age 22-79 years. There was an

excellent correlation between the two measures (r = 0.95 by linear regression) with slope and intercept near 1.0 and zero, respectively. The coefficient of variation for the two measures was 4.1% in non-obese and 3.7% in obese (the group divided at BMI 30 kg/m^2). In the same group of subjects we also compared results with body fat measures using DEXA and bioimpedance. The coefficient of variation for the two measures was 3.6% in non-obese and 3.9% in obese. Also, in this comparison there was an excellent correlation between the two measures (r = 0.92 by linear regression) with slope and intercept near 1.0 and zero, respectively. Taken together these methodological data strongly suggest that determination of body fat mass using either the formula based on BMI[5] or bioimpedance, is accurate and gives a very small error when compared with the gold standard DEXA method.

Condition	BMI kg/m^2	Fat cell volume picolitres	Fat cell number x10^{10}
Before gastric banding	42.2±1.0	890±28	8.4 ±0.5
After gastric banding	34.6±1.3	617±38	8.5±0.6
p-value by Student´s paired t-test	<0.0001	<0.0001	0.98

Table 2. Effects of gastric banding on BMI, fat cell volume and total fat cell number before and 2 years after surgery on obese subjects. The weight loss was 18 ±11 %. Values are mean ± SEM.

DNA extraction

Purified adipocytes were stored at -80 degrees in 25-35ml aliquots. Frozen adipocytes were placed into plastic bags and mechanically disrupted with a hammer. One ml of digestion buffer (500mM NaCl, 50mM Tris.Cl pH 8.0, 125mM EDTA pH 8.0, 2.5% SDS, 0.5mg/ml proteinase K) per 8ml of adipocytes was added to the plastic bag and the sample incubated at +55 degrees overnight (12hrs). The samples were then extracted with equal amounts of

volume of phenol/chloroform/isoamylalcohol. This procedure was repeated until no more protein was seen in the interphase between the two layers. A further extraction was performed using chloroform only. DNA was precipitated by adding half the volume of 7.5M ammonium acetate and two volumes of 95% ethanol to the aqueous phase. DNA was washed thoroughly (4 -6 washes in 70% ethanol for 30-60 mins) before being air dried at +50 degrees. DNA was resuspended in 0.5-1.0 ml DNAse-free pure water and incubated at +60 degrees for several days. DNA purity was confirmed using a spectrophotometer and HPLC analysis.

AMS analysis of ^{14}C content

Purified DNA samples suspended in 1.0-1.5 mL water glass vials were lyophilized to dryness, resuspended in 0.25 mL of 18.2 MΩ DDI water and transferred to quartz AMS combustion tubes. Each glass vial was rinsed 3x with 0.25 mL of 18.2 MΩ DDI water and the rinsate was added to resuspended DNA in the quartz combustion tubes. The DNA and rinsate were again lyophilized to dryness. AMS isotopic standards Oxalic Acid II and ANU sucrose underwent the same drying and transfer as the DNA. Excess copper oxide (CuO) was added to each dry sample, tubes were evacuated and sealed with a H_2/O_2 torch. Tubes were placed in a furnace set at 900 C for 3.5 h to combust all carbon to CO_2. The evolved CO_2 was purified, trapped, and reduced to graphite in the presence of iron catalyst in individual reactors[7]. Magnesium perchlorate water traps were used during graphite production[8]. Large CO_2 samples (> 500 μg) were split and $\delta^{13}C$ was measured by stable isotope ratio mass spectrometry. Graphite targets were measured at the Center for Accelerator Mass Spectrometry at Lawrence-Livermore National Laboratory.

Few of the DNA samples were large enough to get $\delta^{13}C$ splits, but those measured ranged from –21 to –23 per mil, values similar to the whole tissues. We used a $\delta^{13}C$ fractionation correction of –23 ±2 for all samples. Corrections for background contamination introduced

during AMS sample preparation were made following the procedures of Brown and Southon[9]. All ^{14}C data are reported in units of percent modern carbon (pMC) with $\delta^{13}C$ fractionation correction. The supplemental information expresses ^{14}C data in other nomenclatures, as decay corrected $\Delta^{14}C$ following the dominant convention of Stuiver and Polach[10] and as $F^{14}C$ post-bomb fractionation corrected data[11]. The $\Delta^{14}C$ convention established for reporting radiocarbon data in chronological and geophysical studies was not developed to deal with post-bomb data, but it is the most common pending the adoption of $F^{14}C$ as a standard nomenclature for post-bomb data[11].

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