APOE ε3 is an energy-thrifty allele – Studies on energy intake, fat storage and energy expenditure in gene targeted replacement mice

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Abstract

Apolipoprotein E (APOE), a constituent of lipoproteins, is suggested to have pleiotropic functions including regulation of adipocyte differentiation and food intake. Of the three human *APOE* alleles, the *ε3* allele is most common, although it evolved from the ancestral *APOE ε4*. Evidence suggests that the worldwide distribution of the *APOE ε3* allele may be a result of adaptive evolution, although the underlying reasons are not yet understood. In this study, we investigated whether the *APOE ε3* allele may be associated with more efficient food conversion and fat storage and thus provide an advantage over the *ε4* allele under certain conditions. Targeted replacement mice expressing the human APOE3 were heavier, ate more and exhibited a higher dietary energy yield compared to APOE4 mice. Fat mass and the expression of genes involved in triglyceride synthesis in adipose tissue were increased in APOE3 versus APOE4 animals, whereas leptin expression was lower, indicating reduced satiety. Energy expenditure was similar, but APOE3 mice spent more time running and covered longer running distances than APOE4 mice in running wheel experiments. Higher expression of Ucp and Fabp4 in skeletal muscle emphasized elevated energy dissipation and mitochondrial utilisation of fatty acids as fuel substrates in APOE4 mice. Our data suggest that APOE3 has the potential to efficiently harvest dietary energy, accumulate fat in adipose tissue and give higher endurance with lower energy loss in skeletal muscle compared to APOE4. We thus propose that APOE ε3 is an energy-thrifty allele compared to ε4, which appears to be energy-dissipative.

Significance statement

The human Apolipoprotein E (APOE) is a polymorphic gene with three major alleles, *ε4, ε3* and *ε2*, of which *ε4* is a mortality factor in the elderly and an independent risk factor for age-related diseases. Positive selection of the *ε3* allele may underlie its worldwide distribution and the high frequency among different populations. However, age-related disease risks associated with *ε4* are unlikely to have played a significant role in the majority of human evolutionary history. We suggest that *APOE ε3* carriers have the potential to efficiently harvest dietary energy and accumulate fat in adipose tissue and to express a high level of physical activity especially in times of scarce food supply, rendering *ε3* an energy-thrifty allele compared to *ε4*.

Introduction

Since the 1970s apolipoprotein E (APOE) has been known as a constituent of lipoproteins including HDL, VLDL and chylomicrons (triglyceride-rich lipoproteins, TRL) (1), enabling reverse cholesterol transport and TRL remnant clearance. The majority of APOE is synthesized in the liver, but many other cell types produce significant amounts such as adipocytes, macrophages and glial cells, indicating multiple roles of the protein. These functions include antiatherogenic effects through promotion of cholesterol efflux in macrophages (2, 3), neuronal repair and synaptogenic activity (4, 5), as well as adipocyte differentiation and lipid storage in adipose tissue (6). Interestingly, APOE is also expressed in the hypothalamus and olfactory bulb, suggesting involvement in appetite and regulation of food intake (7, 8).

Human *APOE* is a polymorphic gene with the three major alleles *ε2, 3* and *4* distinguished by two single nucleotide polymorphisms (SNPs) in codons 112 and 158 of exon 4 (rs429358C>T, rs7412C>T) that lead to two amino acid changes. Of the resultant APOE proteins (APOE4, E3 and E2), APOE4 is the ancestral isoform carrying arginyl residues defined by both codons (9). It is estimated that *APOE* ε*3*, and subsequently *APOE ε2*, evolved from *APOE ε4* more than 200,000 and no more than 80,000 years ago, respectively, due to subsequent exchanges of arginine to cysteine at the two codons (10). In terms of worldwide distribution, the *ε3* allele is most common, with frequencies of 55-91%, followed by *ε4* (5-41%) and *ε2* (0-15%) (11) and may be subject to positive selection (10, 12). However, possible selective explanations for the higher frequency of the derived *ε3* allele are not fully understood. *APOE ε4* allele frequency declines with age (13), which is attributable to an increased risk for age-related diseases (> 60 y) such as cardiovascular and Alzheimer's disease (14, 15). However, such age-related disease risks are unlikely to have played a significant role in the majority of human evolutionary history, when up to 200 years ago, average life expectancies worldwide were less than 40 years, and the majority of individuals died from infectious diseases (16). Alternatively, varying responsiveness to nutritional factors may have also contributed to the successful worldwide distribution of *APOE ε3*.

In the present study we explore the hypothesis that APOE variation influences energy metabolism, food conversion and fat deposition. Our experiments using APOE targeted replacement mice reveal a higher gain in body weight and fat mass, but also a higher level of voluntary exercise in APOE3 compared to APOE4 mice. In addition, we investigated potential underlying molecular mechanisms in adipose tissue, plasma and skeletal muscle and identified potential target genes differentially modulated by *APOE* genotype.

Results

In the present study we investigated differential body weight gain, fat storage efficiency and energy expenditure in APOE3 and APOE4 targeted replacement mice fed experimental diets with either a low or high fat content.

Body weight, food intake and conversion ratio

APOE3 mice exhibited significantly higher body weights on both experimental diets relative to their APOE4 counterparts (**Figure 1A**). On the low fat diet APOE3 mice showed a final mean of 25.7 g compared to 22.6 g for APOE4. Similarly, after 10 months of high fat feeding APOE3 mice reached a body weight of 45.1 g compared to 37.4 g for APOE4. These differences in body weight were also clearly visible on the phenotypic level, where APOE3 mice appear more obese than APOE4 mice on the same diet (**Figure 1B**). Food intake was modestly higher in APOE3 compared to APOE4 mice (2.45 vs. 2.33 g/d) on the low fat diet, and increased significantly with high dietary fat content in APOE3 (to 2.59 g/d), compared to no increase in APOE4 animals (2.32 g/d) (**Figure 1C**). As a result, APOE3 mice had a significantly higher energy intake, suggesting a higher preference for dietary energy and fat compared to APOE4 mice.

Food conversion ratio (FCR) calculated as food intake per body weight gain declines with nutrient density and food digestibility due to improved utilisation of dietary energy. APOE3 mice showed a lower FCR on both experimental diets (65.0 vs. 76.8 on low fat diet; 19.5 vs. 25.8 on high fat diet) compared to their APOE4 counterparts, suggesting a more efficient utilisation of nutrients and dietary energy in APOE3 mice (**Figure 1D**).

Fat storage and synthesis in adipose tissue

Abdominal adipose tissue (AT) volume was determined by micro computed tomography (micro-CT) in mice fed on the high fat diet (**Figure 2A**). Relative to body weight, APOE3 mice had 15-25% higher volumes of total AT (112.3 vs. 90.1 mm^3/g), visceral AT (77.3 vs. 59.8 mm^3/g) and subcutaneous AT (35.0 vs. 30.4 mm^3/g) compared to APOE4 mice. Thus, we next investigated the (mRNA) expression of genes involved in lipid storage and synthesis in white adipose tissue (WAT), revealing significant differences in *Fasn*, *Dgat1* and *Fiaf* expression. Fatty acid synthase (*Fasn*) is a key factor in fatty acid synthesis from acetyl-CoA and malonyl-CoA, and diacylglycerol acyltransferase 1 (*Dgat1*) catalyzes the final stage of triacylglycerol synthesis using activated fatty acids. Thus, the upregulation of both genes in WAT would be expected to increase triglyceride synthesis and fat storage in adipocytes. In APOE3 mice, relative mRNA levels of *Fasn* and *Dgat1* were higher on the low fat diet compared to APOE4 (2.12 vs. 0.44 for *Fasn*; 1.07 vs. 0.74 for *Dgat1*). Transcript levels significantly dropped in response to a high fat feeding (**Figure 2B, C**) in APOE3 mice (to 0.39 and 0.58), whereas no significant

changed occurred in APOE4 mice (0.61 and 0.70). A similar pattern was observed for *Fiaf*, which is an inhibitor of lipoprotein lipase (LPL) activity. LPL mediates the hydrolysis and release of fatty acids from triacylglycerols in circulating lipoproteins, and thus promotes fat storage in WAT. Significantly lower *Fiaf* expression in WAT (1.00 vs. 2.37) (**Figure 2D**) and liver (0.87 vs. 1.98, data not shown) would promote fat storage and increase fat mass in APOE3 compared to APOE4 mice. Increasing dietary fat content leads to increased *Fiaf* mRNA levels in APOE3 mice and unchanged levels in APOE4 mice. These data indicate that APOE3 mice preferentially store lipids in adipose tissue and respond to increments of dietary fat, while APOE4 mice show comparatively less flexibility and no significant adjustment to dietary fat.

The adipocyte derived hormone leptin is an indicator of body fat and important regulator of food intake. Interestingly, APOE3 mice showed significantly lower leptin mRNA levels (0.69 vs. 1.01) and reduced plasma levels (2.8 vs. 5.0 ng/ml) on the low fat diet although body weight and fat mass were significantly higher than in APOE4 animals (**Table 1**). The lower leptin expression may explain the higher food intake by APOE3 mice. Plasma leptin levels increased dramatically in response to high fat diet in APOE3 and also APOE4 mice, but food intake was similar or even higher, which likely indicates reduced leptin receptor sensitivity after 10 months of an energy dense high fat diet.

Energy expenditure and expression of involved proteins in skeletal muscle

APOE3 mice, given the opportunity to exercise on a running wheel for 24 h, spent significantly more time exercising (14 vs. 9 h) and covered twice the distance compared to APOE4 mice (6.01 vs. 2.94 km, p=0.053) (**Figure 3A**). Energy expenditure (EE) was significantly elevated during exercise in APOE3 and APOE4 mice (**Figure 3B**). In addition, there was a non-significant trend for lower EE in APOE3 than APOE4 mice independent of running activity (two-way ANOVA p=0.100). These results indicate that APOE4 mice show less physical activity, but similar or possibly higher levels of EE compared to APOE3 mice, suggesting more dissipation of energy. Next, we determined protein levels of uncoupling protein (Ucp) and fatty acid binding protein 4 (Fabp4) by Western blotting in skeletal muscle (**Figure 3C**). Uncoupling proteins enable the separation of oxidative phosphorylation from ATP synthesis in the mitochondria, resulting in energy loss by heat production. Although its main tissue source is brown adipose tissue, Ucp expression is also relevant in skeletal muscle by promoting fatty acid oxidation (17, 18). Fabp4 binds fatty acids in skeletal muscle and as such provides them as fuel substrates for mitochondrial activity. Both proteins, Ucp and Fabp4, were higher expressed in APOE4 mice, suggesting increased fatty acid oxidation and higher energy loss in skeletal muscle. Thus, these data support the hypothesis that APOE3 mice exhibit an energy-thrifty phenotype and efficiently retain dietary lipids, while APOE4 mice show a comparatively dissipative phenotype with a

higher degree of mitochondrial uncoupling and utilisation of fatty acids as fuel substrates instead of storing fat in adipose tissue.

Effect of dietary restriction (DR) on energy expenditure and running wheel activity

Total EE and running wheel activity were significantly elevated in dietary restricted mice, a phenomenon which has been observed before (19). Importantly, EE was higher in APOE4 DR mice (**Figure 3E**) and values of total EE differed significantly between APOE3 and APOE4 DR mice (39.5 *± 1.7* vs. 44.6 *± 1.9* kJ/(h*kg$^{0.75}$)). In contrast, APOE3 mice showed significantly higher running performance. Relative to their *ad libitum* fed counterparts, APOE3 mice spent 4.5-fold more time for running with 6.7-fold longer distance (**Figure 3D**), while APOE4 mice increased running time and distance 2.8-fold and 3.7-fold, respectively. Taken together, restriction of dietary energy intake leads to higher increase in energy expenditure in APOE4 mice, but, at the same time, to a higher increase in wheel running in APOE3 mice. It appears that the energy-thrifty phenotype associated with APOE3 is even more pronounced during DR.

Discussion

This study provides important experimental data relating APOE genotype to lipid storage, energy expenditure and running activity, and we suggest that this has possibly played a role in the predominance and geographical distribution of the *APOE ε3* allele. Our data indicate that APOE3 could provide selective advantage over APOE4 by more efficiently storing dietary energy as fat, by lower energy dissipation through curbing mitochondrial uncoupling and by higher running endurance. We thus propose that *APOE ε3* is an energy-thrifty allele compared to *ε4*, which appears to be energy-dissipative.

In our APOE TR mice we observed a significant association of APOE genotype with body weight already at 12 weeks of age. Relative to APOE4, APOE3 mice were heavier independent of dietary fat content, which persisted over the whole study period of 10 months. Similar results with APOE3 mice gaining higher body weight after two to four months of high fat diet feeding were previously reported (20, 21). Interestingly, *Apoe* knockout mice seem to be resistant to diet-induced obesity (22) and introduction of the human *APOE ε3* in wild type mice accelerates body weight gain (23). However, human data are rare and partly inconsistent; while *APOE ε3* compared to *ε4* has been associated with higher BMI and body weight in children and adults (24-28), others found no significant differences (29, 30). Furthermore, determination of the *ε3* allele requires simultaneous consideration of two SNPs (rs429358C/T, rs7412C/T) and is therefore not sufficiently represented on

common genotyping platforms. This could be one reason why *APOE* genotype has thus far not emerged as modulator of obesity measures such as abdominal fat, waist-to-hip ratio or BMI in large scale genome wide association studies (31-34). In addition, the effect of the *APOE ε3* allele on BMI can be considered rather minor, as BMI, like other complex traits, is influenced by multiple genetic and environmental factors. Accordingly, APOE4 mice were not protected from diet-induced obesity, but APOE3 mice gained more body weight on both low and high fat diets.

Our APOE3 mice ate more than APOE4 mice, especially on the high fat diet, suggesting increased appetite and a stronger preference for dietary fat. Leptin, an important regulator of food intake that is derived from adipose tissue (35), was lower in APOE3 mice and possibly associated with reduced satiety on the low fat diet. With increasing dietary fat content and obesity, leptin levels were substantially increased in all mice. Calorie intake was however not reduced, pointing to a reduction of leptin sensitivity. In addition, genes involved in lipid synthesis in adipose tissue (*Fasn, Dgat1*) were significantly up-regulated in APOE3 mice. The inhibitor of triglyceride release from circulating lipoproteins (*Fiaf*) (36) was significantly lower on both low and high fat diets, which would promote plasma clearance and storage of lipids in the WAT. These data suggest a stronger emphasis of energy storage in the form of lipids in adipose tissue in APOE3 than APOE4 mice, which is reflected by the higher percentage of body fat. Due to the fact that relative body weight gain calculated as the food conversion ratio was higher in APOE3 than APOE4 mice, energy expenditure, involved proteins and level of physical activity were evaluated. We here present for the first time comparative data on energy expenditure and voluntary wheel running in APOE TR mice, with the observation that APOE3 mice ran with more endurance than their APOE4 counterparts. In spite of being more active, APOE3 mice expended similar or even less energy than APOE4 mice, an effect that reaches significance upon dietary restriction. We suggest that the higher degree of energy dissipation in APOE4 mice is due to increased uncoupling of mitochondrial respiration through Ucp. Furthermore, increased utilisation of fatty acids as oxidative substrates is indicated by up-regulation of Fabp4 in skeletal muscle. Importantly, the relative increase of wheel running upon dietary restriction was significantly higher in APOE3 mice despite the already higher activity level under *ad libitum* fed conditions compared to APOE4. Food shortage-induced hyperactivity is a widespread phenomenon in many animal species (19, 37). According to the fleeing-famine hypothesis, it is considered an adaptive mechanism that facilitates migrations from depleted environments towards habitats with potentially energy-rich resources (38). Such a behavior has also been observed in humans, for instance in mobile hunter-gatherer groups who readily move to new areas to avoid starvation (39).

Taken together, our data suggest that carriers of APOE3 have the potential to more efficiently harvest dietary energy, to accumulate fat in adipose tissue and to express a high level of physical activity. This might render *APOE ε3* a candidate for a thrifty allele under conditions of scarce food supply. The 'thrifty gene hypothesis' originally proposed by Neel (1962) suggests that genes for efficient food collection and fat deposition were selected in human ancestors that allowed them to survive periods of famine, while if food is continuously available these genes are disadvantageous because they render modern humans obese and susceptible to civilization diseases (40). *APOE ε3* does not meet the criteria of Neel's 'thrifty genes' as it is not detrimental in food abundance and is moreover considered the 'healthy allele', associated with lower mortality risk in the elderly compared to *ε4* (41, 42). Through the course of human evolution, the effects mediated by the *ε3* allele may have provided a competitive advantage over *APOE ε4*. It is interesting to note that the *ε3* allele is estimated to have arisen about 220,000 years ago (10), coinciding with the emergence of anatomically modern humans in Subsaharan Africa at a time when a cold and dry climate prevailed and food resources may have been fluctuating and limited (43). The *ε3* allele and its phenotype of metabolic plasticity may have allowed Homo sapiens to cope better with such adverse conditions. This may also include increased mobility and the propensity to migrate, escaping from barren areas to more pleasant climates. Since then *ε3* has become the most frequent *APOE* allele. However, the current worldwide distribution of *ε3* is not uniform; the allele appears to be particularly common (>84%) in populations that have had a long-established agricultural economy, such as those of the Mediterranean region (11). This may reflect changes in diet and subsistence strategies that began about 12,000 years ago. It has been suggested that this Neolithic transition from hunting-gathering to agriculture introduced prolonged episodes of mass starvation due to recurrent crop failures and diminished dietary diversity (39). The repeated cycles of famine experienced by subsequent generations of farmers are thought to have provided sufficient selective pressure for the accumulation and the spread of thrifty alleles (39). However, these arguments have been criticized as being flawed; especially the effects of famine on the human gene pool are considered exaggerated (44, 45). Future studies are needed to evaluate whether and to what extent the present-day *APOE ε3* frequency pattern has indeed been shaped by dietary instability in human (pre-)history.

Finally, we suggest that APOE4 abets energy 'unthriftiness' by increased mitochondrial fatty acid oxidation in muscle instead of accumulation in adipose tissue as energy reserve. The elevated energy dissipation of the APOE4 mice may also partly explain the non-random global distribution of the human allele. *APOE ε4* frequencies display a curvilinear relationship with geographical latitude, i.e. frequencies are high near the equator, decrease in mid-latitudes and then increase again from about 35° towards the poles (46). We have recently shown that the *APOE ε4* allele is associated with higher

vitamin D serum levels in northern Europeans; the enrichment of the allele in populations who live at high latitude is considered an adaptive response that protects against vitamin D deficiency in environments with low UVB radiation (47). Additionally the clustering of *ε4* in regions with very hot or cold temperatures, where metabolic expenditure for thermogenesis is elevated (46), supports the role of *ε4* as an energy-dissipative allele. The temperature adaptability of APOE4 in combination with a possible increased propensity to migrate upon food shortage associated with APOE3 may have contributed to the current geographical distribution of *APOE* alleles.

Materials and Methods

APOE targeted replacement mice and diets

The mouse study was approved by the local animal ethical committee and complied with German regulations of animal welfare. Forty-one homozygous APOE3 and APOE4 targeted replacement (TR) mice were purchased from Taconic Europe A/S (Ry, Denmark) at the age of 4-6 weeks. APOE TR mice were originally developed in the laboratory of Nobuya Maeda (48, 49) as previously described (47). After delivery and two weeks of acclimation, mice were assigned to different dietary regimens with purified semi-synthetic diets (Ssniff, Soest, Germany) (**Table 2**) according to individual body weights (equal means of body weight per group). For induction of diet-induced obesity, five APOE3 and APOE4 mice, respectively, were fed an energy dense high fat and sugar diet (HFD) and were compared to mice fed with a low fat diet (LFD, n=5). To mimic marginal food supply, 5-6 mice of each APOE genotype were dietarily restricted (DR) and fed 70% of the HFD amount eaten by corresponding *ad libitum* fed counterparts (n=5-6). All mice were held under a 12h light/dark cycle (22-24°C, 55-60% humidity) and had free access to diets (except for DR mice) and fresh drinking water. Food intake and body weight were determined on a daily and weekly basis, respectively. The food conversion ratio was calculated as the ratio of food intake to body weight gain. Mice were sacrificed at an age of 7-10 months, blood and tissues (inguinal white adipose tissue (WAT), skeletal muscle and liver) were collected, snap frozen and stored at -80°C for analyses (except for samples for RNA isolation which were put in RNA*later* (Qiagen, Hilden, Germany) stored at -20°C).

Indirect calorimetry and voluntary running wheel activity

Energy expenditure and magnitude of voluntary exercise of mice were assessed in triplicate measurements after 6 months of high fat diet feeding. Volumes of O_2 consumption (VO_2), CO_2 production (VCO_2) and voluntary running wheel activity were measured using the TSE PhenoMaster (TSE Systems GmbH, Bad Homburg, Germany). Single mice were placed in respiratory chambers for

48 h (including 24 h of adaptation) with an air flow of 0.35 l/min and air sampling every 15 min for O_2 and CO_2 analysis. All respiratory chambers were equipped with a running wheel for voluntary exercise with automatic acquisition of running duration and distance. Energy expenditure (EE) was calculated as follows: EE = (3.941*VO_2 + 1.106*VCO_2)*4.1868/1000 and expressed as kJ/(h*$kg^{0.75}$). Energy expenditure during exercise and recovery (running and not running) were determined.

Micro-computed tomography (micro-CT)

Micro-CT was used to determine the volume of visceral and subcutaneous adipose tissue of high fat mice at the age of 6 months. Four APOE3 and APOE4 mice were anesthetized with Ketamine/Xylazine and the abdominal region between the first and the fifth lumbar vertebra was scanned using a cone-beam *in vivo* micro-computed tomography system (vivaCT 40 Scanner, Scanco Inc., Brüttisellen, Suisse). The scan was performed using the following parameters: energy settings of the X-ray source 45 kVp and 177 µA, voxel size 76 µm, integration time 300 ms, 250 projections per 180°. Sub-cutaneous and visceral adipose tissue volumes were calculated with an algorithm adapted from Lublinsky et al. (2009) (50), which utilized Canny Edge Detection and mathematical morphological operations (http://bme.sunysb.edu/labs/sjudex/miscellaneous.html).

qRT-PCR, Western blotting and ELISA analyses

Relative mRNA levels of fasting induced factor Fiaf (*Angptl4*), letpin (*Lep*), fatty acid synthase (*Fasn*) and diacylglycerol acyltransferase (*Dgat1*) were analysed in adipose tissue and liver. Total RNA of white adipose tissue and liver was isolated using peqGOLD Total RNA or peqGOLD HP Total RNA Kits (Peqlab Biotechnologie GmbH, Erlangen, Germany). RNA quality and concentration was measured using NanoDrop technology (Thermo Scientific, Peqlab Biotechnologie GmbH). Primers for quantitative reverse transcriptase PCR were designed using Primer3 Input software (version 0.4.0): *Angptl4* (Fiaf) 5'-ACTTCAGATGGAGGCTGGAC-3', 3'CCTGTGATGCTGTGCATCTT-5'; *Dgat1* 5'-ACGGATCATTGAGCGTCTCT-3', 3'-GGTCTCCAAACTGCAGAAGC-5'; *Fasn* 5'-GATGGAAGGCTGGGCTCTAT-3', 3'-TGCCTCTGAACCACTCACAC-5'-; *Lep* 5'-TGACACCAAAACCCTCATCA-3', 3'-TCATTGGCTATCTGCAGCAC-5'. One-step qRT-PCR was carried out with the SensiMix™ SYBR No-ROX one step kit (Bioline, Luckenwalde, Germany) and with SYBRGreen detection using the Rotorgene 6000 cycler (Corbett Life Science, Sydney, Australia). Relative mRNA level were calculated with an external standard curve and related to housekeeping gene expression. Following primers were used for housekeeping genes: beta-actin (*Actb*) 5'-GACAGGATGCAGAAGGAGATTACT-3', 3'-TGATCCACATCTGCTGGAAGGT-5'; beta-2 microglobulin (*B2m*) 5'-GTCGCTTCAGTCGTCAGCAT-3', 3'-GTATGTTCGGCTGCCCATTC-5'; eukaryotic translation elongation factor 2 (*Eef2*) 5'-

GCGTGCCAAGAAAGTAGAGG-3', 3'-AAGATGGGGTCCAGGATGAG-5' and cyclophilin A (*Ppia*) 5'-CACCGTGTTCTTCGACATCA-3', 3'-TCCTTTCTCTCCAGTGCTCAG-5'.

Protein expression of Ucp and Fabp4 were analysed in protein lysates of skeletal muscle prepared as described previously (51). Proteins were separated by SDS–PAGE using Criterion™ TGX Stain-Free™ Precast Gels (BioRad, Munich, Germany) and transferred onto a PVDF membrane. Target proteins were identified, using respective primary anti-Ucp (1:1000) or anti-Fabp4 (1:1000) anitbodies and corresponding secondary antibodies (all Abcam, Cambridge, UK). Protein bands were visualized with ECL reagents (Fisher Scientific, Schwerte, Germany) in a ChemiDoc XRS system and band intensities calculated with the Image Lab 4.1 Software (both BioRad).

Plasma leptin levels were measured with the Mouse Leptin Quantikine ELISA Kit (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions.

All data were calculated as means ± SEM (n=4-6). Statistical analysis was performed using SPSS 15.0 (SPSS, Munich, Germany). Data were analysed for normality of distribution (Kolmogorow-Smirnov and Shapiro-Wilk test) prior to student's T-tests. In the absence of normal distributed data the Mann-Whitney-U test was applied. A value of $p<0.05$ was considered statistically significant. Two-way ANOVA was performed for energy expenditure with *APOE* genotype and exercise as independent variables.

Author contributions

PH and GR designed research. PH, JD and AS performed research and analysed data. CCG and GC contributed the micro-CT analysis. PH, AN, JFB, AMM and GR wrote and revised the manuscript.

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Conflict of interest

The authors declare no conflict of interests.

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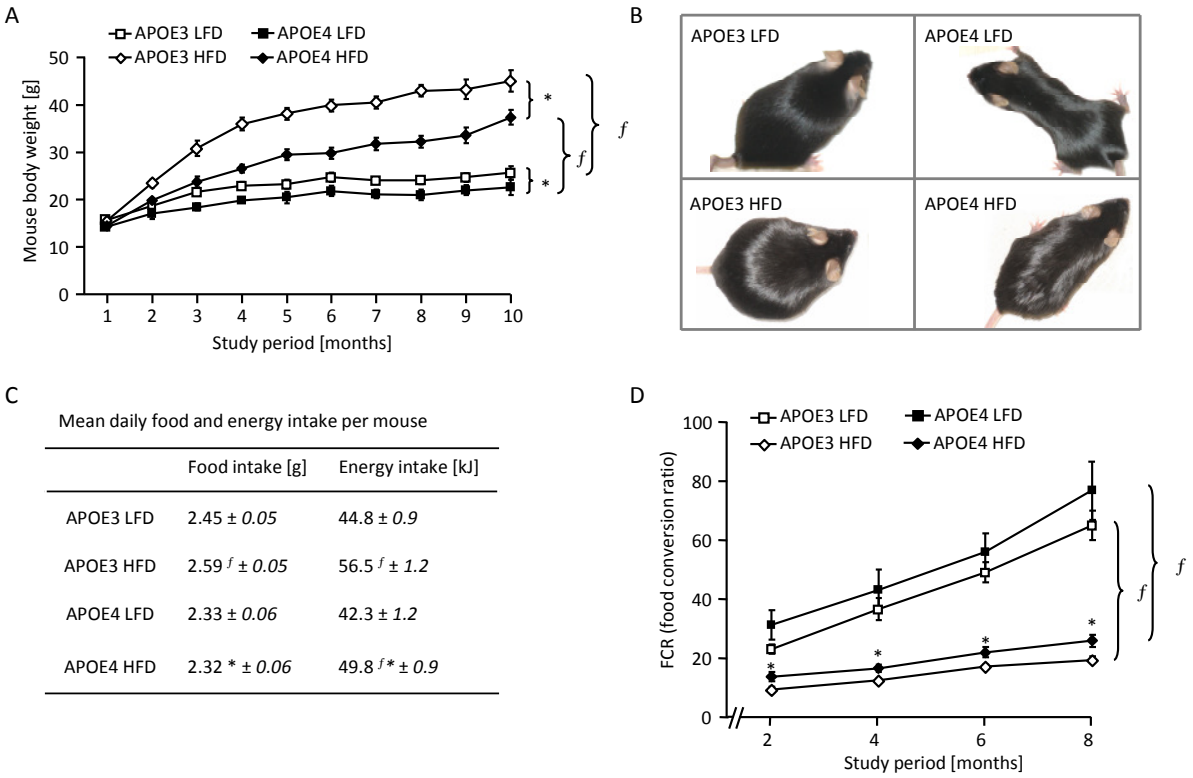
Figures



	Food intake [g]	Energy intake [kJ]
APOE3 LFD	2.45 ± *0.05*	44.8 ± *0.9*
APOE3 HFD	2.59 ^{f} ± *0.05*	56.5 ^{f} ± *1.2*
APOE4 LFD	2.33 ± *0.06*	42.3 ± *1.2*
APOE4 HFD	2.32 * ± *0.06*	49.8 ^{f}* ± *0.9*

Figure 1: Targeted gene replacement (TR) mice expressing human APOE3 exhibit a more obese phenotype, eat more and more efficiently utilise dietary energy compared to mice with an APOE4 genotype. (A) Body weights during a study period of 10 months. Body weights of APOE TR mice are higher on the high fat diet, with APOE3 mice exhibiting significantly higher body weights than APOE4 mice. (B) Adult 12 months old APOE3 mice appear more obese compared to their APOE4 counterparts on both the low and high fat diets. (C) Mean daily food and energy intake were documented for the whole study period of 10 months for APOE3 and APOE4 mice. APOE3 eat more than APOE4 mice with statistical significant difference on the high fat diet. APOE3 mice eat significantly more on the high fat than on the low fat diet. (D) Food conversion efficiency rate was calculated as ratio of food intake and body weight gain. The lower the FCR, the higher is the efficiency to utilize dietary energy. FCR is lower on the high fat diet for both APOE genotypes, though APOE3 mice have lower FCRs than APOE4 mice with statistical difference on the high fat diet. All values are means ± SEM (n=4-5), statistical significant differences between the APOE genotypes are indicated as * and in response to dietary fat content indicated as ^{f}. LFD = low fat diet; HFD = high fat diet

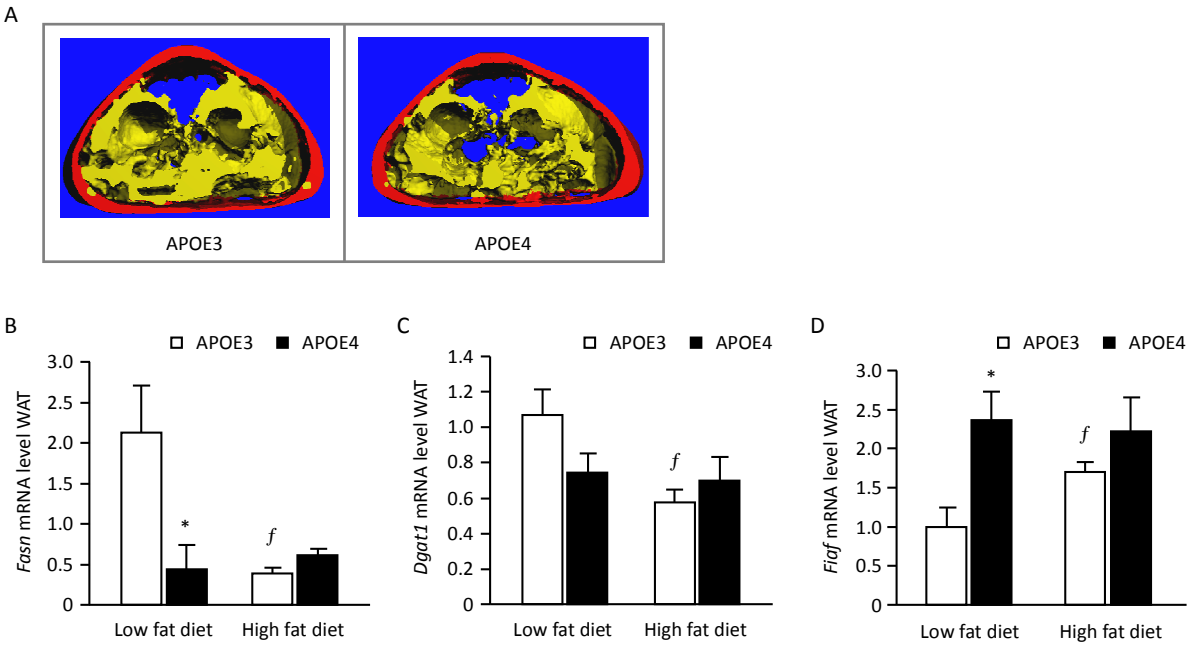
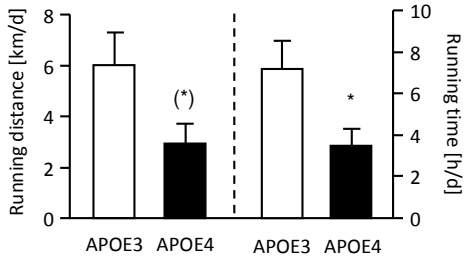


Figure 2: Targeted gene replacement mice expressing human APOE3 have relatively more adipose tissue and show altered expression of genes involved in lipid storage compared to APOE4 mice. (**A**) Volume of total abdominal adipose tissue was measured in APOE3 and APOE4 mice fed the high fat diet for 6 months. Adipose tissue volume was assessed using micro-CT and two images representative for the differences in adipose tissue volume fraction observed are shown. Subcutaneous fat is illustrated as red and visceral fat as yellow area. mRNA level of (**B**) fatty acid synthase (*Fasn*), (**C**) diacylglycerol acyltransferase 1 (*Dgat1*) and (**D**) fasting induced factor (*Fiaf*, a lipoprotein lipase inhibitor) were determined in white adipose tissue (WAT) of APOE3 and APOE4 mice fed a low or high fat diet for 10 months. Target gene expression was measured by qRT-PCR and related to housekeeping gene expression. All values are means + SEM (n=3-5), statistical significant differences (p<0.05 by T-test) between the APOE genotypes are indicated as * and in response to dietary fat content indicated as ^{f}.

A



B

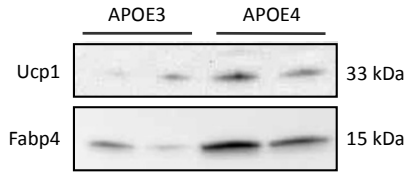
Energy expenditure (EE) during exercise (running) and rest (not running)

	APOE3	APOE4
EE runnig [kJ/(h*kg$^{0.75}$)]	39.4 ± *1.8*	41.6 ± *1.8*
EE not running [kJ/(h*kg$^{0.75}$)]	32.2 ^{f} ± *1.1*	35.4 ^{f} ± *1.6*

Two-way ANOVA for energy expenditure

	APOE	running	APOE x runnning
p-value	0.100	< 0.001	0.771

C



D

Relative increase in wheel running in response to 30% dietary restriction (DR)

	APOE3 DR	APOE4 DR
x-fold change in running distance	6.7 ± *1.3*	3.7* ± *0.4*
x-fold change in running time	4.5 ± *0.7*	2.8* ± *0.3*

E

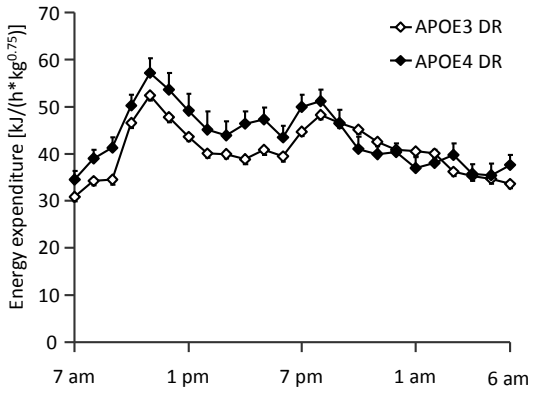


Figure 3: APOE targeted gene replacement mice expressing human APOE3 exhibit an energy-thrifty phenotype but a higher running activity level relative to APOE4 mice. Mice fed a high fat diet for 6 months were placed in indirect calorimetry (IC) cages equipped with running wheels and data were assessed over 24 h. (A) Running distance and time spent for voluntary running per day were higher in APOE3 than APOE4 mice. (B) Energy expenditure (EE) was assessed during exercise (running) and rest phases (not running) and two-way ANOVA was performed for EE depending on APOE genotype (APOE), exercise (running) and interaction of APOE genotype and exercise (APOE x running) as individual variables. While running significantly increases EE in both APOE3 and APOE4 mice, there is a weak trend for lower overall energy expenditure in APOE3 than APOE4 mice. (C) Ucp and Fabp4 protein levels in skeletal muscle are higher in APOE4 compared to APOE3 mice on high fat diet. One representative blot from two animals per APOE genotype is shown. Total protein load per lane was quantified to assure specific target protein regulation. (D) Running performance and (E) total EE of APOE mice was determined in response to 30% dietary restriction (DR) for 4 months on the high fat diet. Upon DR, total EE was significantly higher in APOE4 than APOE3 mice. Running distance and time spent for running per day were significantly increased in both APOE genotypes on DR. The indicated increase in running wheel activity was significantly greater in APOE3 than APOE4 mice.

Values for running distance and time were related to the respective *ad libitum* fed counterparts and given as x-fold change. All values (A-E) are means ± SEM (n=4-6), statistical significant differences (p<0.05 by T-test) between APOE genotypes are indicated as *. (*) indicates a p-value of 0.053. For statistical comparison of EE in (B) significant differences between running and resting phases were indicated as ^{f}.

Table 1: Leptin level in white adipose tissue (WAT, as relative mRNA level) and in plasma of APOE3 and APOE4 mice fed low and high fat diets. Data are means ± SEM (n=3-5), statistical significant differences (p<0.05 by T-test) in response to APOE genotype are indicated as * and in response to dietary fat content indicated as ^{f}.

	Low fat diet		High fat diet	
	APOE3	APOE4	APOE3	APOE4
WAT [*Lep* mRNA level]	0.69 ± *0.28*	1.01* ± *0.22*	2.25 ± *0.92*	2.23 ± *0.51*
Plasma leptin [ng/ml]	2.8 ± *1.0*	5.0 ± *1.5*	37.9 ^{f} ± *5.7*	30.3 ^{f} ± *7.1*

Table 2: Composition of purified semi-synthetic experimental diets

	Low fat diet, LFD	**High fat diet, HFD**
Gross energy [MJ/kg]	18.4	22.1
Nutrients [%]		
Crude protein (from casein)	17.1	17.1
Crude fat	5.1 (from soy oil)	21.2 (from milk fat)
Crude fibre	5.0	5.0
Crude ash	4.2	4.5
Nitrogen free extracts	64.5	49.3
Starch (from corn starch)	39.0	14.5
Sugar (from corn starch)	23.3	32.8
Cholesterol [mg/kg]	-	2.07