

A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations^S

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Abstract The prevalence of obesity has steadily increased over the last few decades. During this time, populations of industrialized countries have been exposed to diets rich in fat with a high content of linoleic acid and a low content of α -linolenic acid compared with recommended intake. To assess the contribution of dietary fatty acids, male and female mice fed a high-fat diet (35% energy as fat, linoleic acid: α -linolenic acid ratio of 28) were mated randomly and maintained after breeding on the same diet for successive generations. Offspring showed, over four generations, a gradual enhancement in fat mass due to combined hyperplasia and hypertrophy with no change in food intake. Trans-generational alterations in adipokine levels were accompanied by hyperinsulinemia. Gene expression analyses of the stromal vascular fraction of adipose tissue, over generations, revealed discrete and steady changes in certain important players, such as CSF3 and Nocturnin.^{¶¶} Thus, under conditions of genome stability and with no change in the regimen over four generations, we show that a Western-like fat diet induces a gradual fat mass enhancement, in accordance with the increasing prevalence of obesity observed in humans.—Massiera, F., P. Barbry, P. Guesnet, A. Joly, S. Luquet, C. Moreilhon-Brest, T. Mohsen-Kanson, E-Z. Amri, and G. Ailhaud. A Western-like fat diet is sufficient to induce a gradual enhancement in fat mass over generations. *J. Lipid Res.* 2010. 51: 2352–2361.

Supplementary key words ω 6 linoleic acid • high-fat diet • generation

The prevalence of obesity and the risk of developing associated diseases have steadily increased across generations over the last few decades. In addition, these events now emerge earlier in life. This epidemic is not attribut-

able to genetic factors as it has occurred relatively recently and is observed in a wide range of human populations. High-fat diets are considered to be obesogenic in that they produce a consistent increase in fat mass that is directly related to the content of the diet and duration of feeding. However, the contribution of dietary fats compared with an excess energy intake in increasing body weight remains controversial, as no major change in the total amount of ingested fats has occurred in the last two decades (1, 2).

In addition to caloric excess, a qualitative issue has emerged as a risk factor for obesity in rodents and possibly in humans; i.e., the disequilibrium in polyunsaturated fatty acid (PUFA) metabolism with a high ratio of linoleic acid (C18:2 ω 6, LA) versus α -linolenic acid (C18:3 ω 3, LNA) (3). Notably, in rodents, reducing this ratio from 59 to 2 under isolipidic, isoenergetic conditions (40% energy as fat) by inclusion of dietary LNA counteracted the enhancing effects of LA on body weight and fat mass, which then became similar to that observed with a chow diet (4). ω 6 PUFAs were more potent than ω 3 PUFAs in promoting adipogenesis (5–7). When combined with high carbohydrate content, a linoleic acid-enriched diet was found to be pro-adipogenic in vivo through cAMP-dependent signaling (8). LA acts through arachidonic acid (C20:4 ω 6, ARA) and prostacyclin, as pups from mice invalidated for the prostacyclin receptor (IP-R) and fed a LA-rich diet exhibit reduced body weight and fat mass compared with wild-type mice fed the same diet (4). Overall, these results emphasize the importance of adipose tissue development

Abbreviations: ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HFD, high-fat diet; IL, interleukin; LA, linoleic acid; LNA, α -linolenic acid; MCP, monocyte chemoattractant protein; PUFA, polyunsaturated fatty acid; SVF, stromal vascular fraction; TNF, tumor necrosis factor; WAT, white adipose tissue.

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in rodents of a high-fat diet combined with a high LA:LNA ratio. Therefore, by analogy to humans, where consumers have been continuously exposed—from in utero to old age—to dietary fats with a high LA content and a low LNA content and in which the prevalence of overweight and obesity has increased within a few generations (9), we decided to set up a nutritional model mimicking a human situation. For this purpose, male and female mice were chronically exposed over four generations to a single Western-like fat diet; i.e., 35% energy as fat with a LA/LNA ratio similar to that found in the most consumed foods. The results show that this condition was sufficient to trigger gradual transgenerational enhancement of the fat mass observed at early and adult ages. In addition to changes in insulin and adipokine circulating levels and to changes in cellularity of adipose tissue, gene expression profiling over generations was used to highlight the major molecular events favoring adipose tissue hyperplasia and metabolic imprinting that lead to an obese phenotype.

MATERIALS AND METHODS

Transgenerational mice and diets

A colony of pure inbred C57BL6/J mice was established by mating four males and four females from the same litter (Charles River, France) that was fed a chow diet. At weaning, pups were either maintained on a chow diet (STD mice) or fed a high-fat diet (ω 6HFD) (HF mice). Both diets contained 21.4% proteins, 3.9% fiber, and 5.7% minerals and ash. The fatty acid composition of the ω 6HFD (35% energy as fat) and chow diet is detailed in supplementary Table I. The ω 6HFD contained a 3.7-fold higher amount of LA (7.9 g per 100 g versus 2.2 g per 100 g) but the same amount of LNA (0.24–0.26 g/100 g) as the chow diet. As shown in **Table 1**, male and female mice were first fed ω 6HFD at weaning and later mated to generate HF0 mice, whereas HF0–HF4 male and female mice were fed ω 6HFD. In this way, HF0–HF4 mice were continuously exposed over generations to the isocaloric, isolipidic diet. The whole population of male and female HF0 adults was mated randomly at 10 weeks of age to give HF1, the first generation (Table 1). Among several possibilities, pups could have been derived from a low body fat gainer male crossed with a low body fat gainer female, or from a high body fat gainer male crossed with a high body fat gainer female. Many additional combinations were also possible. Random mating was chosen on purpose to exclude any selection process. After lactation by HF0 dams, HF1 pups were fed at weaning on ω 6HFD and mated randomly as described above to generate HF2 mice. The same protocol was used to establish generations HF3 and HF4. Reversion experiments were performed by switching HF1, HF3, and HF4 pups to a chow diet at weaning; these mice were respectively termed revHF1, revHF3, and revHF4 mice. Adult male and

female revHF4 mice then fed a chow diet were mated randomly to give birth to the fifth generation of pups that at weaning were fed either the chow diet (termed *std5* mice) or ω 6HFD (termed *hf5* mice) (Table 1). Mice were housed five per cage and body weight was measured weekly for each group of mice from birth to 22 weeks of age. All other measurements were performed with adult mice, i.e., from 8 to 22 weeks of age. At 8 weeks of age, food intake was measured daily for one week. Plasma extraction and adipose tissue dissection were performed as described previously (10). All experimental animal protocols were performed in accordance with the recommendations of the French and European Accreditation of Laboratory Animal Care and were approved by the local experimentation committee.

Lipid analyses of plasma and adipose tissue

After a 12 h fast, blood was collected from 14- and 22-week-old mice ($n = 4$). Plasma and adipose tissue were analyzed for the fatty acid content by direct transesterification as described (11).

Breast milk

Indirect measurement was performed by collecting the stomach content of breast-fed HF1 pups ($n = 4$ for each group). Total lipids of the stomach content were extracted in the presence of 0.02% butylhydroxytoluene before performing gas chromatography (11).

Metabolic parameters

Blood was collected in 22-week-old mice ($n \geq 4$) by heart puncture after a 14 h fast. Then plasma leptin, insulin, total adiponectin, interleukin (IL)-6, tumor necrosis factor (TNF)- α , resistin, plasminogen activator inhibitor (PAI)-1, and monocyte chemoattractant protein (MCP)-1 concentrations were assessed with a Lincoplex assay (Millipore, St Quentin en Yvelines, France).

Morphometric analysis of isolated adipocytes

Epididymal fat pads from 10-week-old mice ($n \geq 3$) were isolated and weighed before cell dissociation for 30 min with collagenase type IV (Sigma Chem.). After filtration (250 μ m), the infranatant was removed by catheter aspiration. In each condition, three pictures of isolated adipocytes per mouse were taken with a light microscope. Measurement of the cell diameter and number was performed as described previously (10).

RNA extraction

Total RNA was extracted from stromal vascular fraction (SVF) cells prepared from periepididymal adipose tissues of 10-week-old mice ($n \geq 3$ per group) using TRI-REAGENT (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions, and then used for cDNA microarray and quantitative PCR.

cDNA microarray

Biological experiments used for expression profiling were independently performed twice. Hybridization was dye balanced to reduce the effects of using two distinct dyes, Cy3 and Cy5. The quality of total RNA was controlled on an Agilent Bioanalyzer

TABLE 1. Experimental protocol to obtain generations of chow diet (STD)- and ω 6HFD-fed mice

	STD	HF0	HF1	HF2	HF3	HF4	revHF1	revHF3	revHF4	<i>hf5</i>	<i>std5</i>
Number of generations on a ω 6HFD	0	0	1	2	3	4	1	3	4	4	4
Diet of parents before and during breeding, pregnancy, and lactation	Chow	Chow	HFD	HFD	HFD	HFD	HFD	HFD	HFD	Chow	Chow
Diet after weaning	Chow	HFD	HFD	HFD	HFD	HFD	Chow	Chow	Chow	HFD	Chow

Abbreviations: HF0–HF4, HFD-fed mice; *hf5*, ω 6HFD-fed fifth-generation offspring of revHF4; HFD, high-fat diet (ω 6HFD); revHF1, HF1 pups fed STD diet at weaning; revHF3, HF3 pups fed STD diet at weaning; revHF4, HF4 pups fed STD diet at weaning; STD, chow diet-fed mice; *std5*, STD-fed fifth-generation offspring of revHF4.

2100 as previously described (12). Pangenomic microarrays were printed using the mouse RNG/MRC oligonucleotide collection (corresponding to 25,299 distinct probes) as previously described (<http://www.microarray.fr/>). Experimental data and associated microarray designs have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under series GSE16613 and GSE16636, and under platform record GPL1456. All calculations were performed with the Bioconductor packages (13) limma (14) and topGO (15). Differentially expressed genes were selected using a Benjamini-Hochberg correction of the *P* value for multiple tests, based on a *P* value of 0.05 or below. Additional parameters corresponded to an average $\log_2(\text{Signal})$ above 6, a $\log_2(\text{Fold change})$ above 0.7 (absolute value) in one of the comparisons.

Quantitative PCR

Reverse transcriptase reactions and quantitative RT-PCR assays were performed as described (16). The expression of selected genes was normalized to that of 18S rRNAs and quantified using the comparative- ΔCt method. Oligonucleotide sequences, designed using Primer Express software (PerkinElmer Life Sciences, Courtaboeuf, France), are available upon request.

Statistical analysis

Data are presented as the mean \pm SEM. Measurements were compared via the two-tailed *t*-test or Kruskal and Wallis nonparametric rank-sum test. Body weight curves were compared by ANOVA analysis for repeated measures using XLstat software, followed by either Bonferroni (for pairwise comparison) or Dunnett (for comparison to STD) post hoc analysis. Microarray experiments were analyzed using the limma package from Bioconductor. The scripts used for the analysis are available in the supplementary data.

RESULTS

Body weight and fat mass across generations

As shown in Table 1 and supplementary Table I, four male and four female C57BL/6J mice from the same litter were mated randomly. At weaning, their pups were fed an LA-enriched diet [termed ω 6 high-fat diet (ω 6HFD)], which contained 35% energy as fat with a LA:LNA ratio \sim 28 (see supplementary data). From weaning up to 8 weeks old, body weight was not affected by the diet. However, when the adult male and female HF0 mice on the ω 6HFD were mated randomly and produced HF1 pups, the body weight of the male mice at weaning became significantly higher (10.9 ± 0.2 versus 9.6 ± 0.6 g, $P < 0.05$; $n \geq 6$ for each generation) than that of the HF0 mice; this weight difference persisted at the adult age. Suckling HF1 pups from both sexes were fed the ω 6HFD at weaning and then mated as above. The difference in body weight both at weaning and at 8 weeks was further increased in HF2 male pups and adults compared with HF1 mice, although no significant weight difference persisted between HF1 and HF2 mice at the adult age (supplementary Fig. 1A).

Regarding fat mass, a large difference in the weight of the epididymal fat pad was already observed across generations at 8 weeks for HF0, HF1, and HF2 mice (Fig. 1A; $n \geq 6$ for each generation). This difference became more evident when comparing HF0 and HF2 mice at 14 (Fig. 1B)

and 22 weeks (Fig. 1C), consistent with the obvious difference in the morphological appearance of STD and HF4 mice at 22 weeks (Fig. 1D). Most importantly, the number of pups per litter was not statistically different: 5.5 ± 0.6 , 5.6 ± 0.5 , 5.0 ± 0.4 , 4.5 ± 0.5 , and 4.7 ± 0.4 for STD/HF0, HF1, HF2, HF3, and HF4 mice, respectively. This finding showed that the ω 6HFD had no impact upon reproduction, thus excluding the possibility that litter size could explain an indirect effect of the diet across generations by increasing the energy intake of suckling pups. When assessed at 8 weeks, no significant difference in the caloric intake could be observed among STD, HF0, HF1, HF2, and HF4 mice ($n \geq 5$; supplementary Table II). Moreover, when HF4 pups were transferred at weaning to a chow diet (Table 1), the food intake of revHF4 mice ($n = 3$) remained similar to that of HF4 and previous generations. Thus, these results exclude the possibility that changes in food and fat intake—across generations early and late in life—explain the transgenerational increase in body weight and fat mass observed at the adult age, suggesting that other mechanisms are implicated. Interestingly, body weight at birth appeared to decrease across generations, but this trend was not statistically significant (supplementary Fig. 1B). The major effect of the ω 6HFD on body weight of STD/HF0 versus HF3 mice took place between the second and third week, suggesting a similar differential effect on fat mass that was not accurately measurable at this very early age (supplementary Fig. 1C).

Lipid profile across generations

PUFA metabolism in the mothers' milk lipids was altered in response to the linoleic acid-enriched diet but then remained similar across generations. The ω 6HFD altered the PUFA composition of milk lipids of HF0 dams. It strongly increased the content of LA and to a lesser extent that of ARA (supplementary Fig. 1IA, B; $n = 4$ for each group). In contrast, it decreased significantly the content of long-chain polyunsaturated fatty acids (LC-PUFA) of the ω 3 series, i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), thus increasing the ARA:DHA and ARA:DHA+EPA ratio from 1.3 to 2.2 and from 1.0 to 1.5, respectively (supplementary Fig. 1IA, C). As for mothers' milk, the ω 6HFD induced an increase in the ω 6 content (LA and ARA) and a decrease in LNA, EPA, and DHA in plasma and adipose tissue lipids at 14 weeks (Fig. 2 and supplementary Table III). All these observations are in agreement with human studies showing that an increase in LA intake leads to stimulation of ARA and/or inhibition of EPA and DHA synthesis (17). The PUFA profile was not different between HF0 and HF4 mice ($n = 4$), showing that no further changes occurred once STD-fed mice had been switched to the ω 6HFD (Fig. 2). Taken together, these results show that the PUFA profile was altered by the diet fat content and that a steady state was then observed across generations. As expected, changes in the PUFA content induced by the ω 6HFD were reversed in a single generation of mice fed the chow diet (revHF4; $n = 3$). Palmitoleate (C16:1 ω 7) has recently been reported to be an insulin-sensitizing, adipose tissue-derived hormone that

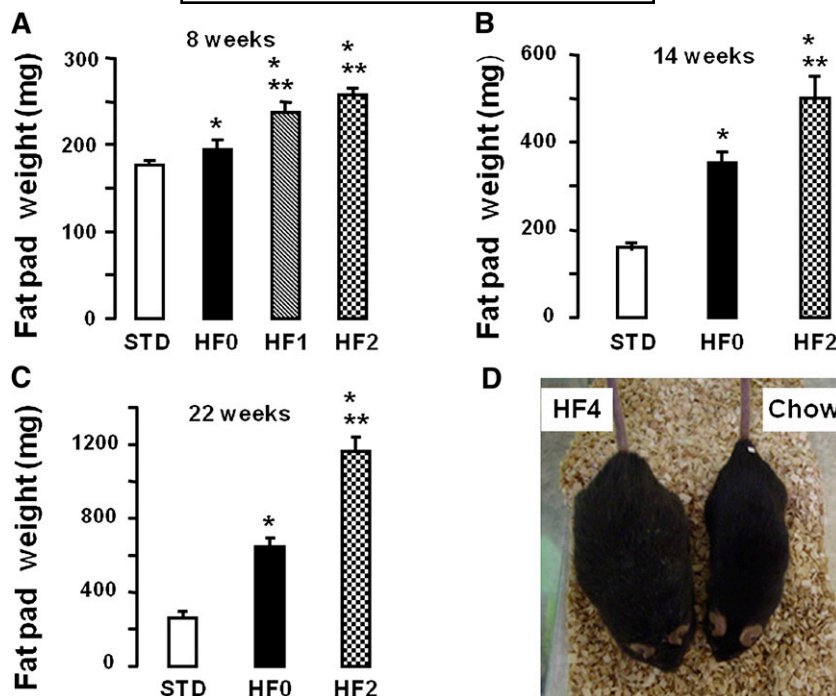


Fig. 1. (A–C) Epididymal fat mass as a function of age. Data are means \pm SEM. * $P < 0.05$ versus STD; ** $P < 0.01$ versus HF0; $n > 6$ for each generation. (D) Morphology of representative male STD after four generations and HF4 mice at 22 weeks old. STD, chow diet-fed mice; HF0–HF4, HFD-fed mice; HFD, high-fat diet ($\omega 6$ HFD).

improves glucose metabolism (18). Palmitoleate levels were regulated in plasma and adipose tissue with a 2- to 3-fold reduction upon initial $\omega 6$ HFD exposure, but no further change occurred across generations (supplementary Table III). The decrease in the $\omega 3$ LC-PUFA status in mice fed the $\omega 6$ HFD had no impact on the DHA concentration in brain phospholipids (supplementary Table III). This finding excludes an essential fatty acid deficiency arising across generations, which would have altered various physiological functions and subsequently affected white adipose tissue (WAT) development.

The adipose phenotype can be partially reversed at later generations

To assess whether further transgenerational effects of the $\omega 6$ HFD could be reversed, HF4 mice were switched at

weaning to the chow diet. In plasma, the percentage of $\omega 6$ PUFAs of revHF4 mice was also similar at 14 weeks old to that of STD mice. The ARA:EPA+DHA ratio increased more than 2-fold in HF4 mice and reversed completely in revHF4 mice. A reversible pattern was also observed for the $\omega 6$ and $\omega 3$ PUFA composition of adipose tissue lipids (Fig. 2 and supplementary Table III). Under these conditions, the body weight of revHF4 mice returned 5 weeks later to that of HF0 mice (Fig. 3A; $n \geq 4$). Importantly, a similar but incomplete reversal was observed for the epididymal fatpad weight (Fig. 3B; $n \geq 6$). Therefore adult male and female revHF4 mice were subsequently mated randomly to give birth to the 5th generation of pups (Table 1). When the litters were fed at weaning either the chow diet (*std5* mice) or the $\omega 6$ HFD (*hf5* mice; $n = 5$ for each group), not only were *std5* mice heavier at weaning

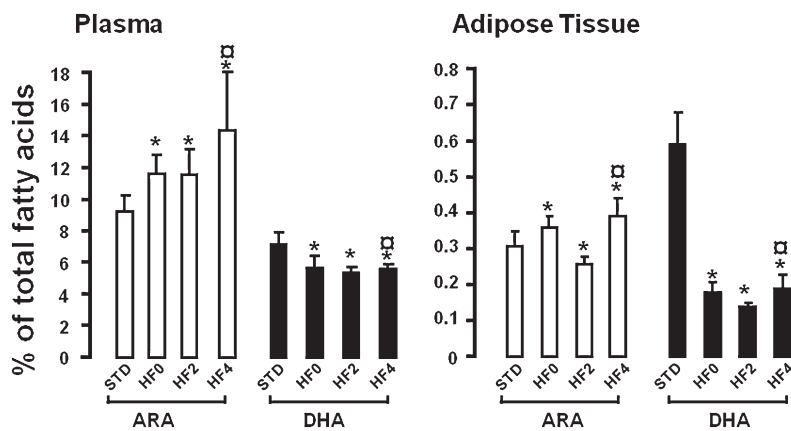


Fig. 2. ARA and DHA content of plasma and adipose tissue lipids across generations (see supplementary Table III for details). *Significant difference versus STD at $P < 0.05$; \square No significant difference versus HF0. ARA, arachidonic acid; DHA, docosahexaenoic acid; STD, chow diet-fed mice; HF0–HF4, HFD-fed mice; HFD, high-fat diet ($\omega 6$ HFD).

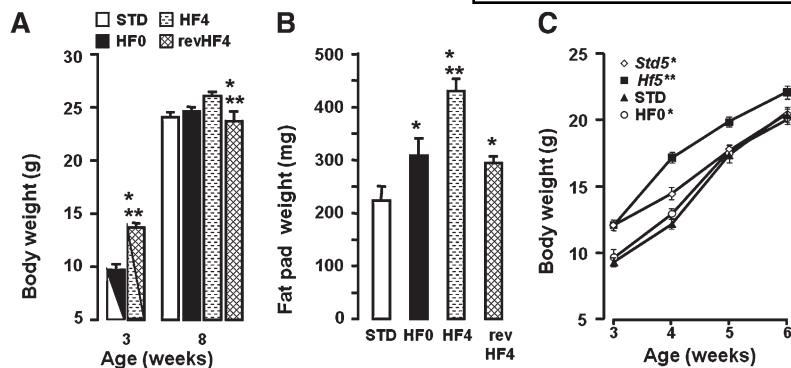


Fig. 3. Reversion of the adipose phenotype. Body (A) and fat pad weight (B) at 8 weeks old. Data are means \pm SEM. * $P < 0.05$ versus STD; ** $P < 0.01$ versus HF0. Differential response of *std5* and *hf5* weaned pups in response to the ω 6HFD (C). For each generation, $n \geq 4$, (A); $n \geq 6$ (B); and $n = 5$ (C). Data are means \pm SEM. * $P < 0.05$ for *hf5* versus *std5*/HF0. HF0–HF4, HFD-fed mice; HFD, high-fat diet (ω 6HFD); *hf5*, ω 6HFD-fed fifth-generation offspring of revHF4; revHF4, HF4 pups fed STD diet at weaning; STD, chow diet-fed mice; *std5*, STD-fed fifth-generation offspring of revHF4.

than STD mice, but a large difference in the rate of weight gain could be observed in *hf5* mice compared with *std5*, as early as the first week after weaning and maintained thereafter (Fig. 3C). It is striking that such a difference was not observed for HF0 mice for which the body weight was similar to that of STD mice from weaning until 8 weeks (supplementary Fig. 1A). The incomplete reversal of the adipose phenotype at later generations suggested that some transgenerational memory had been acquired, allowing revHF4 mice to respond more rapidly than STD mice to the ω 6HFD. This point was further examined at the physiological and cellular levels.

Metabolic changes in plasma across generations

Compared with mice fed a chow diet, those fed the ω 6HFD for 19 weeks after weaning exhibited an increase in the plasma level of most parameters traditionally associated with the metabolic syndrome (i.e., TNF α , resistin, insulin, leptin, and MCP-1) in the first, second, and third generations, while adiponectin and IL-6 levels remained rather constant. Surprisingly, this inflammatory signature was almost reversed at the fourth generation. TNF α , resistin, IL-6, MCP-1, and leptin levels became dramatically reduced compared with those observed in HF3 mice and became similar to those of STD mice (Fig. 4; $n \geq 4$). Fasting insulin levels followed a pattern similar to that of cytokines but remained significantly higher than those of HF0 mice. These observations indicate that, despite the fact that glycemia in HF4 mice appeared normal at 22 weeks old (151 ± 30 mg/dl for HF4 versus 170 ± 30 mg/dl for STD mice), continuous exposure to the ω 6HFD led to a sustained increase in plasma insulin levels, which strongly

suggests the emergence of insulin resistance of adult animals at later generations.

Cellularity and cell subpopulations of adipose tissue across generations

Exposing pups at weaning to the ω 6HFD led within seven weeks to changes in adipose tissue cellularity (Fig. 5; $n \geq 3$). HF0 mice exhibited an increase in the percentage of very small adipocytes (~ 20 μ m) and a slight increase in that of large adipocytes (40–70 μ m). This finding suggests recruitment of adipocyte precursors from the SVF and a trend toward adipocyte hypertrophy. The percentage of small adipocytes (20–30 μ m) increased in HF1 mice, suggesting further recruitment of adipocyte precursors. However, in HF3 mice, a substantial change in cell hypertrophy occurred, with a shift to large-sized adipocytes (50–70 μ m) and the emergence of a population of severely hypertrophied adipocytes (80–100 μ m). Cell hypertrophy became dramatic for HF4 mice, in which two additional populations of large-size adipocytes, 50–70 and 70–130 μ m, could be observed. A fairly large proportion of small adipocytes (20–40 μ m) remained detectable in HF4 compared with HF0 mice, suggesting that adipocyte recruitment was still taking place at the fourth generation.

When HF4 mice were switched at weaning to a chow diet, the pattern of adipocyte size distribution of revHF4 mice was dramatically altered after seven weeks as both populations of large-size adipocytes could no longer be observed. The values for STD, HF0, HF4, and revHF4 mice were 35.4 ± 15.4 , 35.8 ± 17.9 , 48.8 ± 29.1 and 41 ± 11.5 μ m for the mean adipocyte size, whereas the percentage of cells above 50 μ m in diameter was 12%, 20%,

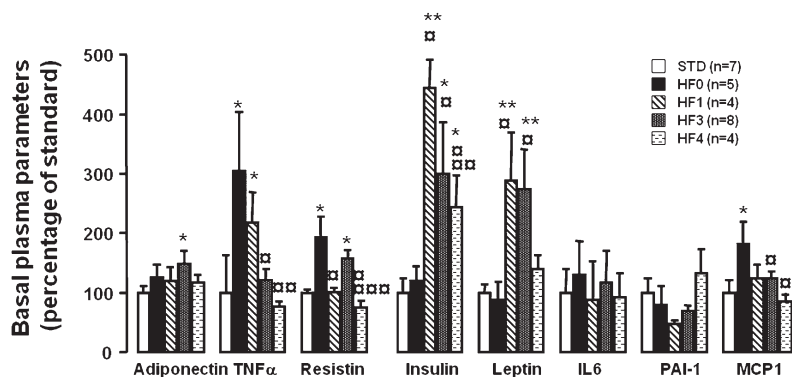


Fig. 4. Changes in insulin and adipokine circulating levels across generations at 22 weeks. Values are means \pm SEM. * $P < 0.05$ versus STD; ** $P < 0.01$ versus STD; \square $P < 0.05$ versus HF0; \blacksquare $P < 0.05$ versus HF1; ▨ $P < 0.05$ versus HF3 ($n \geq 4$). HFD, high-fat diet (ω 6HFD); HF0–HF4, HFD-fed mice; IL, interleukin; MCP, monocyte chemoattractant protein; PAI, plasminogen activator inhibitor; STD, chow diet-fed mice; TNF, tumor necrosis factor.

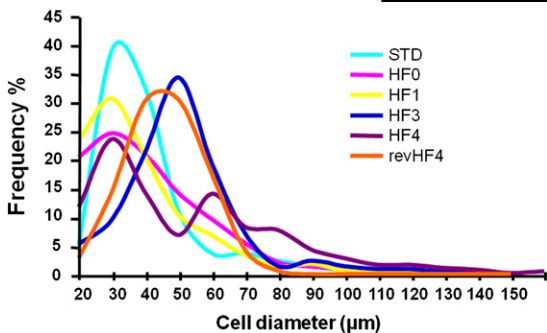


Fig. 5. Adipocyte size distribution across generations. The frequency of distribution was analyzed using the adipocyte fraction of pooled fat pads from at least three mice at 10 weeks old as described in “Materials and Methods.” HFD, high-fat diet (ω 6HFD); HF0–HF4, HFD-fed mice; revHF4, HF4 pups fed STD diet at weaning; STD, chow diet-fed mice.

40%, and 22%, respectively, suggesting “remnant” adipocyte hypertrophy.

To gain insight into recruitment of adipocyte precursor cells, we analyzed by qRT-PCR the expression levels of preadipocyte markers. No significant enrichment was found in the stromal vascular fraction of adipose tissue of HF0–HF3 mice compared with STD mice with respect to Pref-1, PPAR β/δ , C/EBP β , FAT/CD36, or aP2/FABP4 (not shown). These results suggest that the hyperplastic growth of fat tissue implicated progenitor cells were more immature than preadipocytes and able to replenish the pool of preadipocytes. To characterize progenitor cell populations that could ultimately contribute to the formation of new adipocytes, a comparative FACS analysis of stromal vascular cells of epididymal fat tissue was carried out in STD, HF0, HF1, and HF2 mice ($n = 8$) at 4, 8, and 22 weeks of age by using cell surface-specific markers of various cell lineages known to be present in this cell fraction. No difference could be found in the proportion of Sca1⁺/CD34⁺ cells representative of stem cells and immature progenitors or in that of CD45[−] and CD31[−] cells representative of the hematopoietic and endothelial lineages, respectively (not shown). Clearly, we cannot exclude changes in the proportion of the minor subpopulation of CD34⁺/Sca1⁺/CD24⁺ cells, which have been shown very recently to behave *in vivo* as early adipocyte progenitors (19).

Gene expression analysis of stromal vascular cells

Expression profiles of the SVF of adipose tissue were established for the different generations of mice fed the ω 6HFD ($n \geq 3$ per group). Two independent experiments were carried out, and the identified probes were kept for subsequent analysis. To rule out possible contamination by adjacent tissues, a preliminary experiment was performed to directly compare the profile of SVF with those of testis and epididymis. This led to a list of 2,366 distinct SVF-specific probes, which was subsequently used for further analysis. We then selected from this list about 108 transcripts divided into four groups (supplementary Table IV), based on a linear model proposed by Smyth in the

limma package from Bioconductor (20). Probes were selected based on average $\log_2(\text{Signal})$ above 6, a $\log_2(\text{Fold change})$ above 0.7 (absolute value), and an adjusted P value below 0.05 for the following comparisons: HF3 versus HF0, and HF4 versus HF0. This initial analysis identified 46 transcripts (groups 1 and 2) that varied after three or four generations fed the ω 6HFD. Because we felt that such a criterion was too restrictive and may not identify genes involved in the partial reversion of the phenotype (Figs. 3 and 5), we selected additional transcripts for which the expression differed due to the change in regimen from the chow diet to the ω 6HFD and vice versa. The final list was established based on the following comparisons: (HF0+HF1+HF3+HF4) versus (STD+revHF1+revHF3+revHF4). Such transcripts were expected to correspond more precisely to transcripts affected negatively or positively by the change in regimen (groups 3 and 4).

The four lists of genes that varied along generations or after modification of the regimen were then inspected using Ingenuity Pathways (<http://www.ingenuity.com>) to reveal any significant enrichment of functional networks. These genes were then further directly analyzed to identify transcripts with known functions in adipocyte biology as discussed below (Table 2). Genes showing a significant difference but with no published evidence for involvement in adipose biology were not pursued further.

Genes regulated across generations

As hyperplasia of adipose tissue took place over generations, we anticipated a generation-dependent increase in the expression of growth factors. Colony-stimulating factors (CSF) are proteins necessary for survival, proliferation, and differentiation of hematopoietic progenitors, but the specificity of these growth factors is rather promiscuous. For instance, overexpression of CSF-1 (M-CSF) *in vivo* led to a dramatic increase in adipose tissue growth in rabbits through WAT hyperplasia (21), whereas invalidation of the CSF-2 (GM-CSF) gene reduces inflammation in adipose tissue of C57 BL/6J mice in response to a high-fat diet (22). Of interest, our data show that expression of CSF-3 (G-CSF) increased over generations in mice fed the ω 6HFD (validation by qRT-PCR, supplementary Fig. III). These results strongly suggest a role for CSF-3 in stimulating growth of adipocyte progenitors and in enhancing macrophage recruitment. Despite the fact that upregulation of Nocturnin (Ccrn41) did not reach statistical significance, the phenotype of the ω 6HFD-fed mice led us to assess its expression by qRT-PCR. As shown in supplementary Fig. III, this circadian deadenylase, the deletion of which confers resistance to diet-induced obesity, showed increased expression over generations, suggesting a pro-adipogenic role in fat tissue formation by promoting nuclear transport of PPAR γ (Ref. (23) and unpublished observations). There was a sustained, decreased expression of interferon γ (Ifng) over generations, suggesting that the ω 6HFD altered the number of interferon γ -producing T cells, which should in turn decrease the expression of inflammatory genes (24). However, upregulation of other genes favoring recruitment of neutrophils

TABLE 2. Genes displaying adiposity status, impairment of angiogenesis, and inflammation

Gene Identification Number	Gene Symbol	Description	Mode	Fold Change
NM_008337	Ifng	interferon γ	Tg	0.61
NM_009971	Csf3	colony stimulating factor 3 (granulocyte)	Tg	2.12
NM_031168	Il6	interleukin 6	Tg	2.56
NM_024435	Nts	neurotensin	Tg	2.00
NM_011347	Selp	selectin, platelet	Tg	2.05
NM_009605	Adipoq	adiponectin, C1Q and collagen domain containing	Rg	0.48
NM_009627	Adm	adrenomedullin	Rg	0.80
NM_011784	Agtr1	angiotensin receptor-like 1	Rg	0.58
NM_019764	Amotl2	angiomin like 2	Rg	0.66
NM_007643	Cd36	CD36 antigen	Rg	0.45
NM_010228	Flt1	FMS-like tyrosine kinase 1	Rg	0.72
NM_026730	Gpihbp1	GPI-anchored HDL-binding protein 1	Rg	0.56
NM_010612	Kdr	kinase insert domain protein receptor	Rg	0.48
NM_013599	Mmp9	matrix metalloproteinase 9	Rg	0.58
NM_009221	Snca	synuclein, α (Snca), transcript variant 1	Rg	0.76
NM_011587	Tie1	tyrosine kinase receptor 1	Rg	0.61
NM_008215	Has1	hyaluronan synthase1	Rg	2.04
NM_031167	Il1rn	interleukin 1 receptor antagonist (Il1rn), transcript variant 1	Rg	1.71
NM_011111	Serpib2	serine (or cysteine) peptidase inhibitor, clade B, member 2 (Serpib2) / PAI-2	Rg	1.43
NM_016687	Sfrp4	secreted frizzled-related protein 4	Rg	2.16
NM_009311	Tac1	tachykinin 1	Rg	1.72
NM_009421	Traf1	Tnf receptor-associated factor 1	Rg	1.48

Abbreviations: Rg, genes regulated by the ω 6HFD; Tg, genes regulated across generations; Tnf, tumor necrosis factor.

and monocytes was noted. Expression of the P-selectin (Selp) gene, reported to be upregulated in HFD-induced obese mice, increased with generations, suggesting local platelet activation and formation of monocyte-platelet conjugates. These cell-cell interactions could then contribute to local inflammatory processes (25). The tendency toward additional proinflammatory pathways was also suggested by enhanced expression over generations of neurotensin (Nts), which is known to participate in the activation of the NF- κ B signaling pathway. Although its expression is increased over generations, the contribution of interleukin 6 (IL-6) secreted from stromal vascular cells should be low compared with that of adipocytes then recovered in blood (Fig. 4), but its local involvement in developing insulin resistance cannot be excluded.

Genes regulated by regimen

Among the genes affected by diet, genes encoding the VEGF receptor-1 (Flt1) and angiopoietin-1 receptor (Tie1), the expression of which is mainly present in the SVF of adipose tissue, were downregulated. Both receptors bind ligands that are known to promote angiogenesis during excessive WAT development in different strains of mice fed a high-fat diet (26, 27). The VEGF receptor-2 (KDR) gene, encoding an early marker of endothelial cell differentiation, was also decreased (28). Interestingly, the APJ receptor (Agtr1), which is part of the apelin/APJ-R signaling system that plays a critical role in the development of a functional network in WAT (29), is equally decreased in the ω 6HFD-fed mice. Last, but not least, expression of adiponectin (Adipoq), which has been reported to promote proliferation and function of endothelial cells (30),

was also decreased. Unexpectedly, the glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (Gpihbp1), a gene highly expressed in WAT and involved in the lipolytic processing of chylomicrons, appeared downregulated by the regimen (31). In all these cases, the variation was equivalent between HF0 and HF3 or HF4, indicating that these modifications were caused by the regimen. Collectively, these data strongly suggest that hypoxia should prevail under such conditions.

In accordance with these observations, which favor the possibility that decreased gene expression on the ω 6HFD may impair angiogenesis, the genes encoding angiomin-like 2 (Amotl2), which is assumed to regulate, like angiomin, the migration of endothelial cells (32) and adrenomedullin (Adm), a potent vasodilator and antioxidative peptide, were downregulated (33). In addition to adrenomedullin, downregulation of the α -synuclein (Snca) and CD36 genes, which may prevent excessive reactive oxygen species (ROS) production (34, 35), was observed. The modulation of extracellular matrix components via the production of metalloproteinases is an important regulator of fat tissue remodeling. The metalloproteinase 9 (Mmp-9) gene, known to be expressed at higher levels in preadipocytes than in adipocytes, decreased over generations in accordance with its downregulation in nutritionally induced obesity of C57BL/6J mice (36).

As expected, chronic exposure to the ω 6HFD led to upregulation of genes implicated in the hyperplastic/hypertrophic development of adipose tissue. The glycosaminoglycan hyaluronan is an essential component of the extracellular matrix that increases adhesion and retention of monocytes in adipose tissue in diet-induced obese

C57BL/6J mice (37). Accordingly, the expression of hyaluronan synthase 1 (Has1), known to be present in adipocytes and preadipocytes, increased with the ω 6HFD. Of note, the increased expression of the precursor Tachykinin 1 (Tac1) suggests enhanced production of substance P, which has been reported to promote proliferation of preadipocytes (38). Regarding adipocyte hypertrophy, the overexpression of PAI-2 (Serpinb2) over generations is consistent with the fact that PAI-2^{-/-} mice fed a high-fat diet exhibit a lower adipocyte size and higher adipocyte number than wild-type mice (39), and suggests that PAI-2 overexpression promotes excessive WAT development. Reports have implicated Wnt signaling pathway components as proteins that maintain the preadipocyte compartment and inhibit adipogenesis. Interestingly, the gene expression of secreted frizzled-related protein 4 (sFRP4) became elevated with the ω 6HFD (validation by qRT-PCR, supplementary Fig. III). As sFRP4 antagonizes the Wnt signaling pathway (40), this strongly suggests a stimulating effect on WAT development. Adrenomedullin, the expression of which decreased over generations, has been proposed to be antiadipogenic (41). If it were so in vivo, this will also favor adipogenesis. With respect to the emergence of insulin resistance, upregulation of interleukin-1 receptor antagonist (Il1rn) and TNF α receptor-associated factor 1 (Traf1) with a regimen appears to be important as the respective blockade of Il-1 and TNF α signaling pathways improved insulin sensitivity in diet-induced obesity (42, 43).

DISCUSSION

We and others have pointed out the detrimental effects in humans caused by a dramatic change in the fatty acid composition of dietary fats at a time when the quantitative changes in fat consumption were not observed. Notably, at a time where overweightness and obesity have steadily increased over generations in most industrialized countries, consumption of LA and ARA has increased. In France, an increase of 250% and 230%, respectively, occurred from 1960 to 2000 (9). During the same period, consumption of LNA has decreased by 40%, resulting in a 4.2-fold increase in the LA:LNA ratio. A similar increase in LA intake has been observed in other countries of the Western world, with a LA:LNA ratio ranging from 10 to 41 for most foods consumed in the US (44). Under isolipidic, isoenergetic conditions, an LA-enriched diet was shown in elderly humans to increase body weight, and the rate of LA accumulation in adipose tissue was associated positively with gain in body weight and, presumably, fat mass (45). In our experiments, LA represented 18% and LNA 0.6% of the total energy intake compared with 5–7% and 0.8–1%, respectively, recommended for humans by expert committees on nutrition. Unfortunately, a significantly higher LA intake and much lower LNA intake are frequently observed in most industrialized countries (3, 9). Our results show that, in a situation of genome stability that is reminiscent of augmentation in the prevalence of obesity observed worldwide, a gradual transgenerational increase in adiposity can occur in mice fed a Western-like fat diet.


So far, studies have been mainly focused on the relationships between diet-induced obesity in female rodents and the development of adult offspring adiposity and associated diseases (46, 47). Herein we chose purposely ad libitum conditions to (i) expose both male and female mice across several generations to a Western-like diet, i.e., 35% energy as fat, whereas in most studies of diet-induced obesity in rodents these figures ranged from 30% to 80% energy as fat and (ii) use at-random breeding of all pups from the same litter, thus excluding, in contrast to a recent report (48), any selection of high or low body-fat gainers, which would have put emphasis on genetic issues and would have been less relevant to humans.

The results show clearly, under conditions minimizing an imbalance between fetal and postnatal environments, a transgenerational increase in body weight and fat mass that was already apparent in pups at weaning and was maintained in adulthood. With respect to adipose tissue development, at any given generation, triggering events associated with the diet can be postulated, as it is known that in utero placenta selectively extracts ARA and DHA from maternal blood. It is thus conceivable that the ω 6HFD will not increase LA levels in fetal blood but rather will increase ARA levels in the maternal and subsequently in the fetal blood (49). According to our data on the fatty acid composition of milk, adipose tissue, and plasma lipids, we assumed after birth a similar contribution of LA in enhancing adipose tissue development owing to the adipogenic role played by its metabolite ARA via prostacyclin at the time where the content of the anti-adipogenic EPA and DHA decreased (4, 7).

Noteworthy, enhanced adiposity occurs over generations through hyperplasia and hypertrophy despite no significant change in food intake in pups and adult mice. Clearly, a progressive decrease in energy expenditure cannot be excluded, although the resistance of C57BL/6J mice to diet-induced thermogenesis has been reported (8). Actually, we observed a trend (not statistically significant) toward higher expression of UCPI in brown adipose tissue, suggesting an increase in energy expenditure over generations. It cannot be excluded that an increase of subcutaneous adiposity contributes to decreased thermogenicity over generations as heat loss is known to increase basal thermogenesis and resistance to diet-induced obesity (50). Regarding the transgenerational hyperplasia of adipose tissue, it may be due to the increased expression of the CSF3 gene across generations that enhanced the proliferation of adipose progenitors. In addition, we suggest that enhanced Nocturnin expression across generations modulates hypertrophy and associated metabolic events through subtle alterations in glucose homeostasis and lipid-partitioning between various organs including adipose tissue, as HF4 mice appear to be a striking mirror image of Nocturnin-null mice fed a HFD. Indeed, compared with wild-type mice, Nocturnin-null mice exhibited similar locomotor activity but an abnormality in weight regulation despite equivalent caloric intake and less heat production. Importantly, these knockout mice also showed improved insulin sensitivity, i.e., similar glucose and lower insulin levels (23).

We observed also impairment in the expression of genes promoting angiogenesis, suggesting increased hypoxic and even highly hypoxic conditions in adipocytes distant from the vasculature, which lead in turn to alterations in adipokine secretion (51) and insulin sensitivity (52). Surprisingly, after a rise in TNF- α , resistin, leptin, and MPC-1 in the first generation, normalization of circulating levels of adipokines occurred in HF4 mice compared with STD mice. Despite fat tissue expansion across generations, leptin levels returned to normal. This suggests that hypothalamic leptin sensitivity had somehow been redefined through transgenerational exposure to the ω 6HFD. Moreover, the decreased levels of inflammatory adipokines could be regarded as adaptive evolution of the autonomic nervous system output. The most persistent dysregulation was that of insulin. In the absence of hyperglycemia, this strongly suggests insulin resistance, which is consistent with a progressive loss in sympathetic inhibition of insulin release over generations. Thus it looks as if an enhanced fat mass was an adaptive response to prevent aggravation of a metabolic syndrome.

Collectively, our data show that continuous exposure to a high-fat diet combined with a high LA:LNA ratio from fetal to adult age over generations triggers a discrete and steady increase in inflammatory stimuli accompanied by enhancement of fat mass that was already observed a few weeks after birth. Most interestingly, in humans, the recent Framingham Heart study pointed out that nonobese offspring with two obese parents had higher C-reactive protein levels compared with offspring with one or no obese parent. Thus offspring with a high risk of developing obesity are characterized at birth by a proinflammatory state (53).

Our results on the incomplete reversal of adipocyte hypertrophy suggest epigenetic changes. To our knowledge, there is no direct evidence that fatty acid nutrition affects epigenetic gene regulation and that epigenetic mechanisms determining overweightness/obesity in an obesogenic environment have any relationship with those observed in malnourished fetuses (54). Among candidate genes, only sFRP4, a member of the Wnt signaling pathway known to exhibit epigenetic regulation through methylation, may be relevant to this observation (55). We speculate that its increased expression over generations should be responsible, at least in part, for the fast "rebound" in body weight observed in *hf5* mice compared with *std5* mice on a high-fat diet. Further studies will indicate whether histone modification rather than methylation is involved in epigenetic changes on continuous exposure to the ω 6HFD. 

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