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Early-Life Exposure to Dietary Large Phospholipid-Coated Lipid Droplets Improves Markers of Metabolic and Immune Function in Adipose Tissue Later in Life in a Mouse Model

Marlou Klein Hazebroek, Annemarie Baars, Mona Mischke, Annemarie Oosting, Evert M. van Schothorst, and Lidewij Schipper**

Scope: Human milk (HM) is considered optimal nutrition for infants, beneficially programming adult health outcomes including reduced obesity risk. Early life exposure to infant formula with lipid droplets closely resembling the structural properties of HM lipid globules (Nuturis) attenuated white adipose tissue (WAT) accumulation in mice upon adult western-style diet (WSD) feeding. Here, the study aims to elucidate underlying mechanisms. Methods and results: Mice are raised on control or Nuturis diets between postnatal days 16–42 followed by either standard diet or WSD for 16 weeks. While the adult body composition of mice on a standard diet is not significantly affected, Nuturis reduced adiposity in mice on WSD. Morphologically, mean adipocyte size is reduced in Nuturis-raised mice, independent of adult diet exposure, and WAT macrophage content is reduced, albeit not significantly. Transcriptomics of epididymal WAT indicate potential beneficial effects on energy metabolism and macrophage function by Nuturis. Conclusion: Reduced adult adiposity by early life exposure to Nuturis appears to be associated with smaller adipocytes and alterations in WAT immune and energy metabolism. These results suggest that early modulation of WAT structure and/or function may contribute to the protective programming effects of the early-life Nuturis diet on later-life adiposity.

1. Introduction

Human milk (HM) is considered the optimal nutrition for healthy development and growth of new-born, due to, e.g., its nutritional composition as well as bioactive compounds that contribute to immunological protection.[1,2] The duration and exclusivity of breastfeeding have been associated with multiple health benefits for infants including lower blood pressure, lower plasma cholesterol, and a lower risk of obesity in later life.[3–6] The latter is suggested to be linked to a reduced weight-for-length ratio in breastfed infants,[7] which reduces the risk for excessive weight and fat mass gain in later life.[8] Potential underlying mechanisms include "nutritional programming," which occurs in developing tissues, and is the physiological setting of metabolism by a stimulus in a critical period that has long-term consequences for functioning of organs.[9]

White adipose tissue (WAT) is a metabolically active tissue that continues to develop after birth and is a key contributor to metabolic health.[10,11] It is the main storage site for fat and responds to feeding and fasting conditions by hypertrophy and hyperplasia.[12] Hypertrophy (i.e., increased cell size) is suggested to underly the increased fat mass that characterizes obesity. This has several adverse effects on tissue health, including decreased insulin sensitivity and metabolic flexibility,[13] changed gene expression patterns,[14] and changes in hormone secretion.[15] Further, hypertrophy of adipocytes is associated with low-grade inflammation in WAT.[16] Adipocytes secrete adipokines, cytokines, and chemokines that increase the recruitment of immune cells, particularly pro-inflammatory and anti-inflammatory macrophages.[17] The pro-inflammatory macrophages can form crown-like structures (CLS) and contribute to dysfunction of adipocytes. Dysfunctional adipocytes secrete pro-inflammatory cytokines, including tumor-necrosis factor alpha, interleukin beta, and chemokines, which activate and attract more macrophages,[18–23] thereby initiating a vicious cycle of inflammation that can eventually affect other tissues, like liver.[24]

M. Klein Hazebroek, A. Baars, M. Mischke, A. Oosting, L. Schipper
Danone Nutricia Research
Utrecht 3584 CT, The Netherlands
E-mail: lidewij.schipper@danone.com
M. Klein Hazebroek, E. M. van Schothorst
Human and Animal Physiology
Wageningen University
Wageningen 6708 WD, The Netherlands
E-mail: evert.vanschothorst@wur.nl

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One of the factors hypothesized to contribute to the long-term beneficial effects of HM is the physical properties of lipid globules. Lipid globules in HM are large, with a volume-based mode diameter of ≈4 μm, and are coated by the milk fat globule membrane (MFGM), a three-layered membrane consisting of phospholipids, sphingomyelin, glycolipids, membrane-specific proteins, and cholesterol. In contrast, due to common manufacturing processes, lipid droplets in standard infant milk formula (IMF) are smaller (mode diameter <0.5 μm) and coated with proteins as main emulsifier.[25] Lipid droplet size and surface area characteristics can influence lipid digestion and absorption kinetics[26–28] affecting bioavailability of lipids and other components to developing organs. Previously, a concept IMF (i.e., Nuturis) was developed in which lipid droplets are large (mode diameter 3–5 μm), and coated with phospholipids derived from bovine milk, thereby more closely mimicking the characteristics of HM lipid globules.[25] In pre-clinical studies, early-life exposure to diets containing Nuturis formula have been associated with lower body weight and fat mass upon a western-style diet (WSD) challenge in later life,[29–32] as well as smaller adipocyte size.[30,33] Recently, Nuturis was also linked to an increased level of lipid metabolism in liver in mice.[34] These studies indicate that the early-life exposure to Nuturis programs metabolism and works through into later life, although the exact mechanisms remain unknown.

Here, we investigated the effects of early-life exposure to Nuturis on adult adipose tissue characteristics, including adipocyte cell size and distribution, macrophage content, and WAT transcriptome-wide gene expression patterns, under standard and obesogenic adult diet conditions. We hypothesized that the physical structure of the lipid droplets in Nuturis would elicit a lower inflammatory profile in adult adipose tissue compared to those in a standard IMF. This was expected to be proceeded by a change in expression patterns of genes related to immune cell infiltration, such as macrophage attractors.

2. Experimental Section

2.1. Animals and Study Design

This study (licence number DAN230) was conducted following the principles of good laboratory animals care and all experimental procedures were approved by an external, independent Animal Ethics Committee (DEC-consult, The Netherlands), under license of the national competent authority, securing full compliance to the European Directive for the use of animals for scientific purposes. Animals were housed in the animal facility of Wageningen University on a 12 h light–12 h dark cycle (light on 06.00 h = Zeitgeber time 0 h), in a temperature- and humidity-controlled room (21 ± 2 °C and 50 ± 5%, respectively). Throughout the study, food and water were available ad libitum, unless specified otherwise. The experimental animals were bred in-house, with $n = 12$ for the experimental groups. The C57BL/6JOlaHsd breeding pairs (Harlan, Gannat, France) were kept on semi-synthetic rodent diet (AIN93G[35]) and were time mated. Two days after birth of the litters (postnatal day [PN] 2), litters were randomized and culled to six pups (at least two males and two females per litter). At PN16, dams and their litters were randomly allocated to a diet containing standard IMF (Control) or Nuturis IMF. After weaning at PN21, dams and female pups were removed, and male pups remained on their respective diet and were housed individually in Mac 2 cages with wood shavings bedding and cage enrichment (shelter and nesting material). Individual housing was required to accommodate indirect calorimetry measurements. At PN42, males were switched to either a Western Style Diet (WSD; 40 kcal% fat) or an AIN93 maintenance variant (AIN93M[35]) until dissection at PN112. This resulted in a total of four different experimental groups (**Figure 1**A). All groups were composed of pups derived from at least four different litters and existed of $n = 12$ male mouse pups in total at weaning age, except for one group in which breeding generated only 10 male mouse pups. Two mice were excluded from the experiment after weaning due to malocclusion, which resulted into the following group sizes: Control-AIN ($n = 10$), Nuturis-AIN ($n = 11$), Control-WSD ($n = 11$), and Nuturis-WSD ($n = 12$).

2.2. Experimental Diets

All diets were semi-synthetic (Research Diet Services, Wijk bij Duurstede, the Netherlands) with macronutrient and micronutrient composition according to the AIN93G formulation.[35] The experimental diets contained 28.3% w/w IMF powder covering the complete lipid fraction and were complemented with protein and carbohydrates to meet rodent nutrient requirements. The detailed composition and processing procedure of the diets had been described previously.[30,31] The experimental diets were provided daily as a fresh dough in the home cage. The experimental diets differed according to dietary lipid structure; lipid droplets in the control diet were small (mode diameter <0.5 μm, and were coated with protein as their main emulsifier, lipid droplets in the Nuturis diet were large (mode diameter: 4.0 μm) and were coated by phospholipids derived from bovine MFGM. The WSD was based on AIN93M with following modifications: higher content of fat (20% w/w fat: 3% w/w soy oil, 17% w/w lard, and 0.1% w/w cholesterol) and sucrose (15% w/w), exchanged with starch content to have identical protein content.

2.3. Indirect Calorimetry

At PN38-PN42 and PN93-PN98 indirect calorimetry measurements were performed (TSE PhenoMaster system, Bad Homberg, Germany) to evaluate energy expenditure, respiratory exchange ratio (RER)—representing substrate usage -, voluntary locomotor activity, and food intake of the mice. After 2 days acclimatization, the third day (24 h) was used for measurements, as published.[36] More details can be found in legend of Figure S1, Supporting Information.

2.4. Body Composition and Dissection

Body weight was monitored twice a week throughout the experiment. Body composition was measured at PN42, PN55, PN70, PN84, and PN98 using an EchoMRI Whole Body Composition Analyser (EchoMRI, Houston, FL, USA). From PN42 onward, food intake was measured twice weekly by subtracting weight of remaining diet from given diet per period. At PN112, mice were

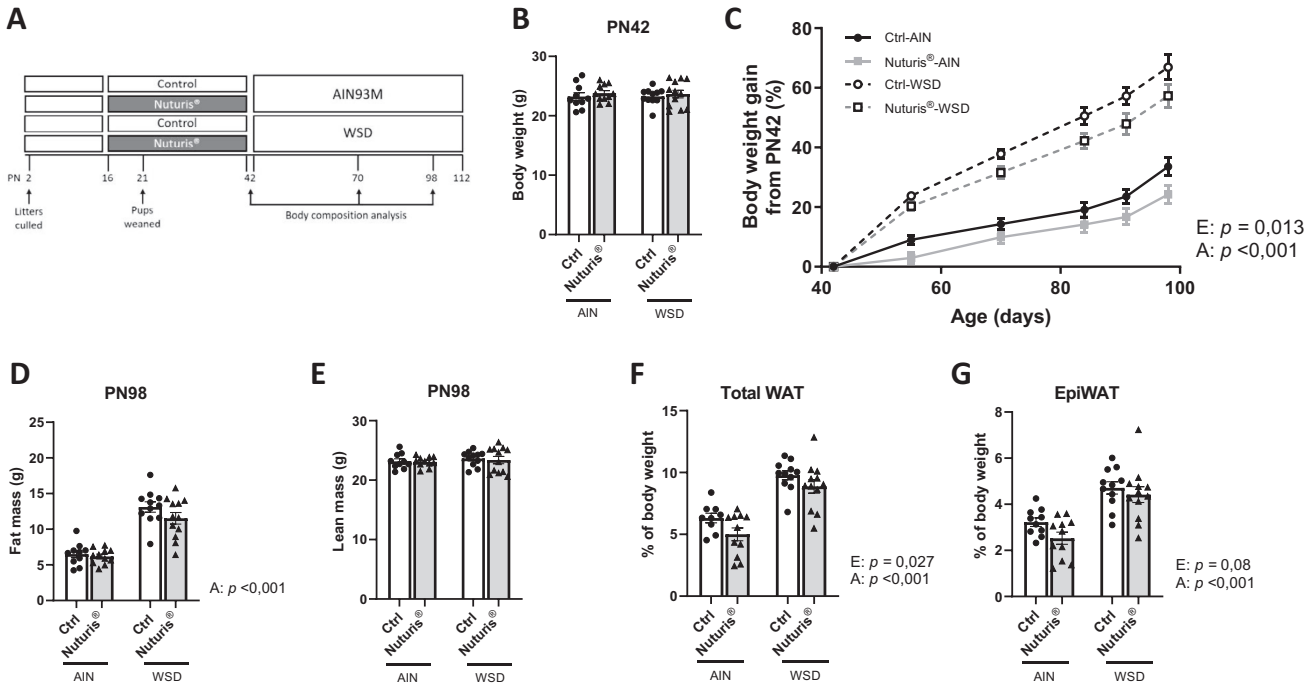

Figure 1. Nuturis feeding in early life reduces body weight gain in later life by affecting fat mass. Experimental setup of the study (A). Body weight of control and Nuturis-fed animals at the start of the adult diet intervention (AIN 93 M) or WSD (B). Body weight gain in % from PN42 until PN98 (C). Fat mass (D) and lean mass (E) of all experimental groups at PN98. Relative total WAT mass (F) and epiWAT mass (G) at sacrifice. Graphs represent n = 10–12 animals per group. p-Values of a two-way ANOVA for factors early-life diet (E) and adult diet (A) are provided in the individual panels.

fasted overnight, and were euthanized the next morning by anesthesia (isoflurane–N_2O–O_2) followed by heart puncture and cervical dislocation. WAT depots (epididymal (epi), retroperitoneal, perirenal, and inguinal) were collected and weighed. Of each animal, one epiWAT depot was snap-frozen in liquid nitrogen and stored at −80 °C and the other was formalin-fixed overnight, and paraffin embedded.

2.5. Adipocyte Cell Size

Formalin-fixed, paraffin embedded epiWAT was sliced into 5 µm sections, deparaffinized, and stained with Mayer's Hematoxylin, followed by immersion with a HCl-ethanol solution (0.125%), a lithium carbonate solution (1%) and Eosin Yellowish (0.1%), as described.[37] Subsequently, sections were dehydrated and mounted. Images were taken with a Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss, Göttingen, Germany) at 10x magnification using a green laser (543 nm) and a detection filter for red wavelengths (560 nm). The study used CellProfiler v2.1.1 (Broad Institute, Cambridge, MA, USA) to identify individual adipocytes and measured their surface area, as published.[38] The diameter (µm) was calculated from the given surface area assuming a spherical shape of adipocytes. For the full procedure, a blinded experiment was used. Random selection of a subset of samples (n = 4 per group) for analyses was performed by a third person not involved in these analyses and based on similar average FM at last timepoint body composition analyses were performed (PN98) per group.

2.6. Crown-Like Structures and Individual Macrophages

To analyze the number of CLS and individual macrophages in epiWAT, 5-µm-wide sections were stained as reported previously.[36] Briefly, sections were deparaffinized, and endogenous peroxidase activity was blocked by immersing the glass slides in a methanol and 3% hydrogen peroxide solution for 30 min. Next, slides were incubated for 30 min with 2% normal goat serum (S-1000, Vector Laboratories, Inc., Burlingame, CA, USA), followed by an overnight incubation (4 °C) with a rat anti-mouse MAC-2 primary antibody (1:5000; CL8942AP, Cedarlane, Ontario, Canada). Subsequently, slides were incubated with secondary biotinylated goat anti-rat IgG antibody (1:200; BA-9400, Vector Laboratories, Inc.) for 60 min. Signal amplification was performed by 60 min incubation with a Vectastain Elite Avidin-Biotin Complex kit (PK-6100, Vector Laboratories, Inc.). Afterwards, visualization was performed by incubating sections with 3.3'-diaminobenzidine solution (1:400; SK-4105, Vector Laboratories, Inc.) for 4 min, and counterstaining for 40 s with Mayer's Haematoxylin. Slides were mounted using VectaMount AQ Aqueous Mounting Medium (H-5501, Vector Laboratories, Inc.). Images were taken with a Zeiss Axioskop 2 microscope with an AxioCamMRc 5 camera (Carl Zeiss). Counting of CLS and individual macrophages in WAT was performed per 1000 adipocytes per animal. For the full procedure, the study used a blinded experiment. Here, the following sample size was used: n = 9 for Control-AIN and Control-WSD, and n = 11 for Nuturis-AIN and Nuturis-WSD groups.

2.7. RNA Isolation

Total RNA was isolated from epiWAT samples using TRIzol reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentrations were measured using NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Breda, The Netherlands). RNA quality was confirmed using an Agilent 2100 Bioanalyzer according to manufacturer's instructions (Agilent, Santa Clara, CA, USA). Samples with RNA integrity number above 8.0 were used, resulting in the following sample sizes: Control-AIN ($n = 7$), Nuturis-AIN ($n = 9$), Control-WSD ($n = 10$), and Nuturis-WSD ($n = 6$).

2.8. Microarray Hybridization and Analysis

Per sample, 100 ng of purified RNA was labeled using the Ambion Whole Transcript (WT) Expression kit (Life Technologies Ltd., Bleiswijk, The Netherlands) and Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, CA, USA). All samples were hybridized to Affymetrix GeneChip Mouse Gene 1.1 ST arrays according to standard Affymetrix protocols. An Affymetrix GeneTitan Instrument was used for hybridization, washing, and scanning of array plates. Quality control and normalization were performed by using Bioconductor software packages integrated in an on-line pipeline.[39] Applying robust multi-array analysis algorithm from the Bioconductor library AffyPLM with default settings, normalized expression estimates of probe sets were computed.[40] Probe sets were redefined and annotated with Entrez gene identifiers using version 19 of the custom CDF provided by Dai[41] resulting in 21 115 assigned Entrez IDs. For each group comparison, signal 2log ratios, fold changes (2log ratio of 1 = fold change of 2) and related significances of change were calculated using intensity-based moderated *t*-statistics (IBMT) implementing empirical Bayes correction.[42] Before further analysis, genes were filtered based on an intensity value of >20 on at least 5 arrays and an interquartile range >0.1, leading to a reduced set of 13 294 genes. Descriptive bioinformatic analysis and visualization of the data were carried out using S.J. Jol's tools for Venn diagrams (https://www.stefanjol.nl/venny) and ClustVis[43] for heat maps, normalizing the data of all samples for the mean of Control-AIN group and applying unit variance scaling for rows, and row-clustering with Euclidean distance and average linkage. To gain functional insights and to identify top canonical pathways associated with the differentially expressed genes ($p < 0.05$, DEGs) of compared conditions, Ingenuity Pathway Analysis (IPA) was performed. Only canonical pathways with a *z*-score above 2 or below −2 were considered, as these represent activated and inhibited pathways, respectively. Disease-specific pathways not deemed relevant to the study setting were excluded.

2.9. Statistical Analysis

Statistical analyses for physiological parameters (Figures 1–3) were performed using IBM SPSS 19.0 (SPSS Benelux, Gorinchem, The Netherlands). These parameters were tested for normal distribution using the Gaussian distribution with the Shapiro–Wilk test. All parameters were normally distributed. Then, multivariate ANOVA was used to determine effects between groups, with early-life (E) and adult diet (A) as factors. For the body weight gain development (Figure 1C), a repeated-measures ANOVA was performed. All data were presented as mean ± SEM, unless otherwise specified. Differences were considered significantly different when $p < 0.05$, and a trend was specified as $p < 0.1$. Statistical analysis of gene expression patterns was given in section Microarray hybridization and analysis.

3. Results

3.1. Nuturis Feeding in Early Life Reduces Body Weight Gain in Later Life by Affecting Fat Mass

Male mice were fed an early-life diet (control or Nuturis) from PN16 until PN42. At PN42 no differences in body weight (Figure 1B), fat mass and lean mass (data not shown) were observed between the groups. Indirect calorimetry measurements from PN38 to PN42 showed no differences between the groups in energy expenditure, respiratory exchange ratio (RER)—representing substrate usage—locomotor activity, and food intake (Figure S1A–D, Supporting Information). In adulthood, mice challenged with WSD showed increased body weight gain compared to AIN-fed mice (Figure 1C). This effect was due to increased absolute fat mass (Figure 1D), as lean mass was similar between all experimental groups (Figure 1E). Indirect calorimetry measurements during PN93–PN98 did not indicate any differences in energy expenditure, RER (Figure S1E,F, Supporting Information), or locomotor activity due to control or Nuturis feeding (data not shown). Cumulative food intake measurements showed a similar caloric intake between PN42 and PN98 (Figure S1G, Supporting Information). Interestingly, in both the AIN and WSD groups, Nuturis-fed animals showed a significantly lower body weight gain compared to controls (Figure 1C). This effect was reflected in total relative WAT mass at dissection at PN112 (Figure 1F). The relative epiWAT weight in WSD-fed groups was increased compared to AIN-fed groups, and a trend ($p = 0.08$) was observed towards a lower epiWAT weight in Nuturis-fed mice (Figure 1G).

3.2. Nuturis Feeding in Early Life Reduces Mean Adipocyte Cell Size

Adipocyte cell size and cell size distribution were determined in Haematoxylin and Eosin stained sections at PN112 (**Figure 2**A–D). The average adipocyte diameter was significantly decreased in mice fed Nuturis in early life compared to mice fed control diet, in both the AIN as well as the WSD groups (Figure 2E). Although mice fed Nuturis demonstrated a higher absolute percentage of smaller adipocytes—representing more smaller cell sizes (up to 60 µm cell diameter) and less larger cells (above 60 µm)—compared to control diet (Figure 2F), no significant differences were observed between groups in different size ranges.

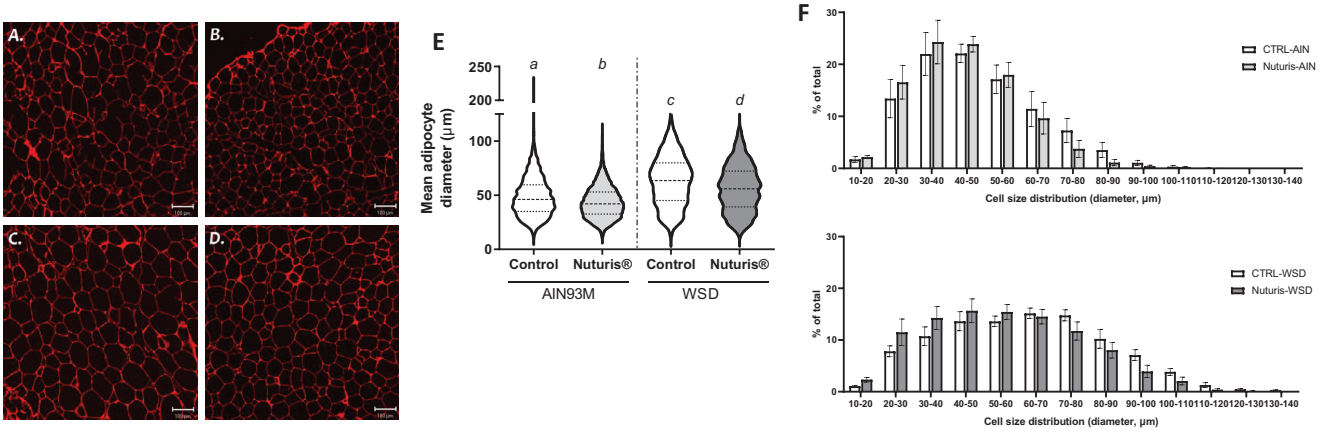

Figure 2. Nuturis feeding in early life reduces mean adipocyte cell size. EpiWAT sections of Control-AIN (93 M) diet (A), Nuturis-AIN diet (B), Control-WSD (C), and Nuturis-WSD (D) were stained with Hematoxylin and Eosin to determine adipocyte diameter. Images were taken with a Laser Scanning Microscope at 10× magnification. The scale bar indicates 100 µm. Mean adipocyte diameter of epiWAT (E) was calculated per group ($n = 4$ animals, 600–1000 adipocytes per animal). Different letters indicate significant differences between groups and were considered significant at $p < 0.05$. Frequency distribution of adipocyte size (F) of control and Nuturis exposed to AIN diet (top) and WSD (bottom).

3.3. Nuturis Feeding in Early Life Modulates Inflammatory Markers in Adult Adipose Tissue

The number of CLS and individual macrophages were assessed in epiWAT at PN112 (**Figure 3**A–D). CLS number and individual macrophages were increased in the WSD groups compared to AIN groups (Figure 3E,F). For both CLS and individual macrophages, there was a trend towards smaller numbers in epiWAT of Nuturis-fed animals compared to controls ($p = 0.0982$ and $p = 0.0524$, respectively, Figure 3E,F).

3.4. Nuturis Feeding Alters Gene Expression Patterns in Adult Adipose Tissue

The molecular signature of adipose tissue was analyzed at PN112 in epiWAT using transcriptome-wide gene expression analyses. The number of DEGs ($p < 0.05$) under adult WSD compared to AIN, representing the effect size, was higher in control than in Nuturis-fed mice ($n = 3771$ and $n = 1832$, respectively; Table S1, Supporting Information). Between Nuturis-fed animals and controls, a moderate difference was observed in the number of DEGs, i.e., 762 genes in AIN compared to 610 genes in WSD groups (**Figure 4**A and Table S1, Supporting Information), yet only 43 genes (3.5%) were differentially expressed in both comparisons (overlap shown in Figure 4A; Table S2, Supporting Information). The top10-regulated IPA canonical pathways for WSD compared to AIN in control groups (Figure S2, Supporting Information) related to generation of precursor metabolites and energy (*TCA cycle, Mitochondrial Dysfunction*), cellular assembly and morphology, as well as immune signaling and functionality, including specifically macrophage-related pathways (*Fcγ receptor-mediated phagocytosis in macrophages and monocytes, production of nitric oxide and reactive oxygen species in macrophages*). For Nuturis, *mitochondrial dysfunction* and *Fcγ receptor-mediated phagocytosis in macrophages and monocytes* were also among the canonical pathways regulated by WSD compared to AIN, however, both *z*-scores were lower in Nuturis, i.e., 2.97 versus 4.61 and 2.68 versus 3.06, respectively, indicating a milder effect. Following the leads of IPA that linked to the physiological outcomes of our study, we next explored the gene expression profiles of the four groups for specific gene sets. Related to the observed trend towards less CLS and individual macrophages by Nuturis, macrophage-related genes in the transcriptomics dataset ($n = 32$, color-coded in Table S1, Supporting Information) were selected.[24,44,45] Displaying these genes in a heat map showed a cluster of genes with relatively higher expression in the Control-WSD group compared to the Control-AIN group (Figure 4B), and $n = 10$ were significantly differentially expressed ($p < 0.05$) for this comparison. This suggests a role of WSD in regulating the expression of these genes. In contrast, the Nuturis-WSD group showed an expression pattern more similar to the Nuturis-AIN and Control-AIN groups for this gene cluster. Indeed, only $n = 5$ genes were significantly higher expressed ($p < 0.05$) for Nuturis-WSD compared to Nuturis-AIN. If we focus solely on the effects of Nuturis (vs Control) on either diet (AIN or WSD), 2 and 1 transcripts, respectively, were significantly altered. Altogether this suggests a moderate, yet beneficial effect of early-life Nuturis exposure on macrophage-related gene transcripts, which is in line with the observed trend-wise lower infiltration of pro-inflammatory macrophages.

A large part of the IPA canonical pathway *mitochondrial dysfunction* relates to oxidative phosphorylation (OXPHOS). Given the reduced adiposity upon Nuturis feeding, the genes of the OXPHOS KEGG-pathway were selected from the transcriptomics dataset ($n = 99$, color-coded in Table S1, Supporting Information), and displayed as a heat map in Figure 4C. Similar to what was observed for the macrophage-related gene cluster, a cluster of genes, mainly related to OXPHOS complex V, the ATPase, was relatively higher expressed in the Control-WSD group compared to Control-AIN, with $n = 11$ genes being significantly higher expressed ($p < 0.05$). The effect of WSD on this complex V gene cluster was less clear in the Nuturis-fed group, which showed an expression pattern more similar to the Nuturis-AIN and

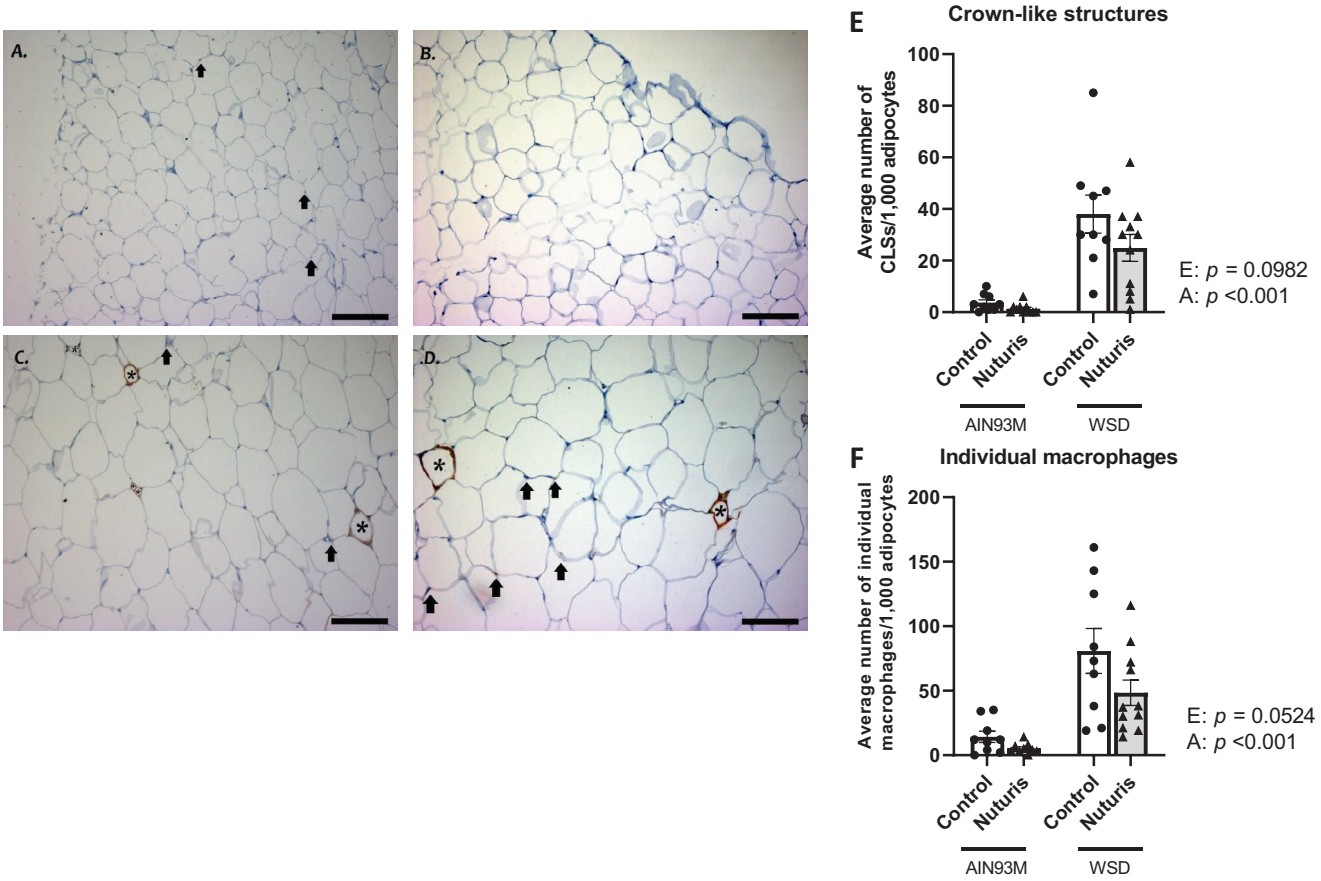

Figure 3. Nuturis feeding in early life modulates inflammatory markers in adult adipose tissue. EpiWAT sections of Control-AIN (93 M) diet (A), Nuturis-AIN diet (B), Control-WSD (C), and Nuturis-WSD (D) were stained with MAC-2 macrophage antibody. Crown-like structures (CLS) are encircled in black. Individual macrophages are indicated by black arrows. Images were taken with a light microscope at 20× magnification. The scale bar indicates 100 µm. The number of CLS (E) and individual macrophages (F) of all experimental groups was counted per 1000 adipocytes. Number of CLS and individual macrophages were calculated per group with $n = 9$ (Control-AIN), $n = 11$ (Nuturis-AIN), $n = 9$ (Control-WSD), and $n = 11$ (Nuturis-WSD). *p*-Values of a two-way ANOVA for factors early-life diet (E) and adult diet (A) are provided in the individual panels.

Control-AIN groups. Only $n = 2$ genes of this complex V cluster were significantly higher expressed ($p < 0.05$) for Nuturis-WSD compared to Nuturis-AIN. Furthermore, the other main gene cluster, relating mainly to OXPHOS complexes I–IV, displayed opposite changes, i.e., the Control-WSD group showed relatively lower expression of most genes compared to Control-AIN, of which $n = 25$ were significantly lower expressed ($p < 0.05$). Focussed on the effects of Nuturis (vs Control) on either diet (AIN or WSD), 2 and 2 transcripts, respectively, were significantly altered, and in both cases, they belong to the OXPHOS Complex I and Cytochrome C subunits with different transcripts.

4. Discussion

In the last decades, it has become clear that not only contemporary diet, but also early-life nutrition, influences adulthood health status. Especially the role of nutrition during the time that metabolic tissues are still in development has received attention, leading to the creation of the term "nutritional programming." Here, we show that early-life feeding with an IMF with large milk-phospholipid coated lipid droplets (Nuturis), more closely resembling the supramolecular structure of lipid globules in HM, induced changes in adipose tissue in adult mice. Nuturis-fed animals showed a significantly lower body weight gain compared to the control group, which was accompanied by decreased total WAT weight independent of adult diet exposure (AIN or WSD). Further, Nuturis feeding reduced epiWAT adipocyte cell size compared to control fed mice. In the same tissue, early-life Nuturis feeding resulted in a change of adipose tissue gene expression patterns, specifically for genes related to energy metabolism, i.e., mitochondrial oxidative phosphorylation, and key macrophage genes after a WSD diet in adulthood. Taken together, these results suggest an improved adipose tissue function in murine adulthood when Nuturis was only given in early life.

The current results confirm and expand on previous findings that early-life exposure to Nuturis mitigates excessive fat mass accumulation during adulthood when subjected to WSD,[29–31] although a fully established energy balance remains to be determined (energy losses via urine, feces, fermentation gasses) as standard indirect calorimetry data suggested no significant differences between the groups. In addition, we find this effect exists upon AIN-feeding during adulthood as well, which

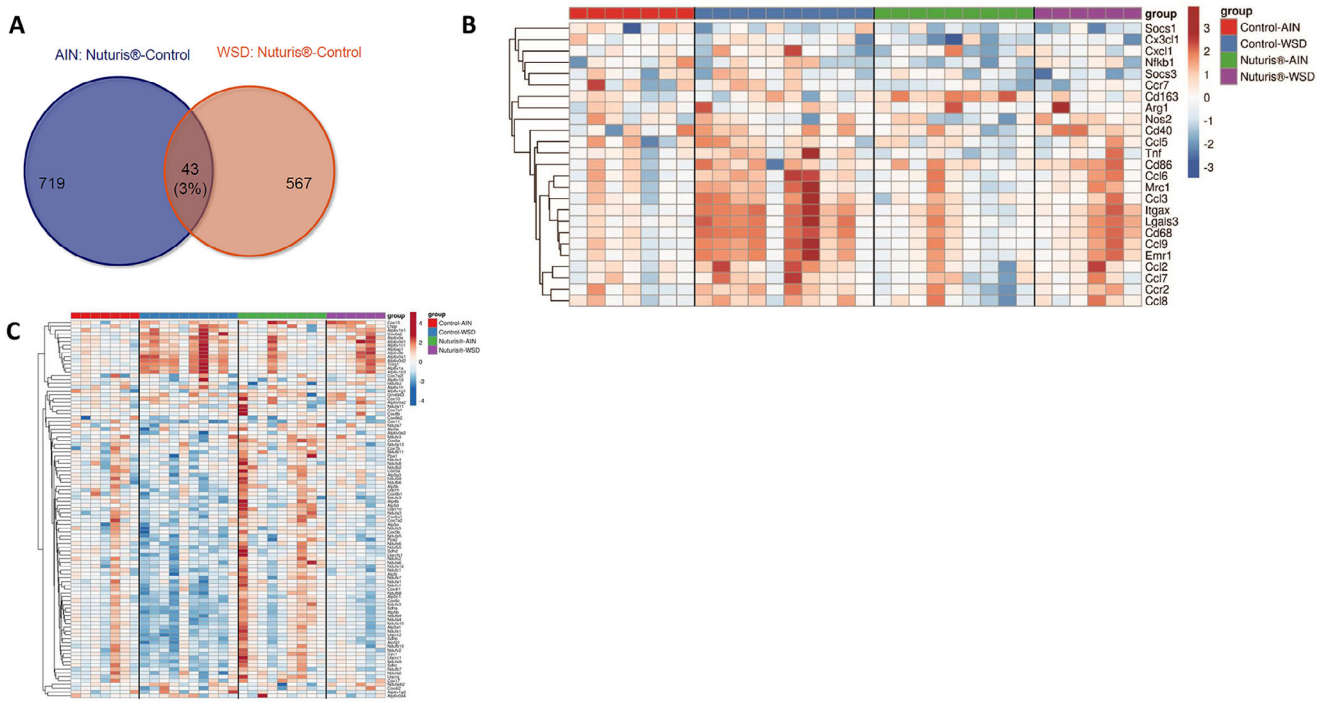

Figure 4. Nuturis feeding alters gene expression patterns in adult adipose tissue. Venn diagram showing the overlap and differences of differentially expressed genes (IBMT regularized *t*-test $p \leq 0{\cdot}05$) in the early-life diet groups (Nuturis vs Control comparison) in mice receiving AIN (93 M) or WSD in later life (A). Relative expression of macrophage-related genes (B) and genes related to oxidative phosphorylation (C) normalized to the mean of Control-AIN group, and shown by a color gradient, with blue indicating lower expression and red indicating higher expression than mean Control-AIN. Unit variance scaling is applied to rows, and rows are clustered using Euclidean distance and average linkage.[30]

strongly suggests that changes in fat mass accumulation are programmed by Nuturis, and not merely "activated" when mice are challenged with a WSD. Previously, we observed that protective effects of Nuturis against adult diet-induced obesity coincided with a reduction in adipocyte size.[30] Indeed, here we also observe smaller adipocytes upon Nuturis feeding. As larger adipocytes are associated with reduced WAT health, early-life Nuturis feeding might have a protective effect on the overall WAT milieu. Indeed, we find trends towards a lower number of both CLS as well as individual macrophages upon Nuturis feeding. To identify CLS and macrophages, we used a general macrophage marker (MAC-2). However, it is well known that different types of macrophages co-exist in WAT. Typically, a distinction is made between classically activated M1 (pro-inflammatory) and alternatively activated M2 (anti-inflammatory) macrophages.[46] However, in recent years, many different shades of macrophages within this binary spectrum were identified.[45,47] Although we did not focus on identifying the type of macrophages in the tissue, the combination with bigger adipocytes in Control versus Nuturis groups points towards a more M1-like phenotype. Further, the fact that CLS are made up of more M1 than M2 macrophages supports this notion.[48] Overall, a lower amount of pro-inflammatory macrophages has been shown to decrease obesity and improve glucose homeostasis (reviewed in Thomas and Apovian[49]). Therefore, our results suggest that early-life exposure to Nuturis induces programming of healthier adipose tissue, which has a beneficial effect into adulthood.

Interestingly, the reduced body weight gain in Nuturis fed groups did not give significant differences in energy expenditure, although lean mass remained similar, and this represents most of the total metabolic tissues. To get insight into whether the molecular profile of WAT could explain the physiological changes by the Nuturis diet, we performed transcriptome-wide gene expression analyses in adult epiWAT depots. Interestingly, our transcriptomics analyses might suggest that differential gene expression by the Nuturis diet for macrophage-related genes depended on the adult diet exposure. Further, we observed a relatively higher expression of genes related to OXPHOS complex V in the WSD groups, which was slightly reduced by Nuturis feeding in early-life. This suggests that, in an adult obesogenic environment specifically, oxidative phosphorylation pathways might be influenced by early-life diet. Indeed, the relative protein expression of OXHPOS subunits, in particular mitochondrial cytochrome c oxidase I subunit (Complex IV) were altered by Nuturis diet in retroperitoneal WAT depots in a previous study with similar study design.[33] Further, Nuturis feeding increased the functional mitochondrial marker maximum respiratory capacity in liver tissues at PN42 (direct effect) and PN98 (as a programmed effect),[50] supporting the gene effects seen on OXPHOS complexes in WAT here. Further functional experiments are needed however to establish the role of early-life nutrition on the mitochondrial electron transport chain function in adulthood. Expression of genes related to macrophage infiltration and inflammatory cell recruitment was increased by WSD exposure, in line with infiltration of macrophages in the epiWAT depot. Mice fed

Nuturis® diet in early-life did show less of these transcriptional changes when challenged with WSD. Taking these molecular observations together, the reduced number of macrophages in epiWAT of Nuturis-fed mice challenged with WSD could suggest that more flexible and less inflamed, smaller adipocytes are seen. Whether the effects seen in adulthood extend into old age and might contribute to an extended healthier phenotype and/or longer survival, remains to be elucidated.

In conclusion, the present study shows that Nuturis feeding in early life reduced cell size and improved metabolic and immune parameters in the adipose tissue of adult mice when challenged with WSD. Early modulation of epiWAT structure and function are hypothesized to contribute to the protective effects of Nuturis on later life health. However, further studies are needed to show if adipose tissue development is instrumental and primary to the observed phenotypic effects.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.K.H. and A.B. contributed equally to this work. The authors' contributions are as follows: A.B., L.S., and A.O. designed the research; A.B., L.S., M.K.H and E.v.S. conducted the research; A.B., L.S. M.M, M.K.H and E.v.S. analysed the data, A.B. and M.K.H. wrote the initial draft manuscript. All authors read and approved the final version of the manuscript. We thank Eefje Engels and Hans Swarts for their excellent expertise and technical assistance with the animal work.

Data Availability Statement

The data that support the findings of this study are available on reasonable request from the corresponding authors.

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infant nutrition, inflammation, metabolism, nutritional programming, obesity

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