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Moderate vitamin A supplementation in obese mice regulates tissue factor and cytokine production in a sex-specific manner

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Abstract

Vitamin A (vitA) regulates obesity, insulin resistance, inflammation, dyslipidemia, and hemostasis through its metabolites retinaldehyde (Rald) and retinoic acid (RA) produced in endogenous enzymatic reactions. Combination of at least 3 of these conditions leads to development of metabolic syndrome (Msyn) and, consequently, type 2 diabetes and/or cardiovascular disease. Although many foods are fortified with vitA, it remains unknown what conditions of Msyn are influenced by moderate dietary vitA supplementation. A family of aldehyde dehydrogenase 1 (Aldh1) enzymes is a key contributor to obesity via sex- and fat depot-specific production of RA in adipose tissue. Therefore, we studied effects of moderate vitamin A supplementation of an obesogenic high-fat (HF) diet (4IU vitA/g and 20IU vitA/g HF diet) on multiple conditions and mediators of Msyn in wild-type (WT, C57Bl/6) and *Aldh1a1*$^{-/-}$ mice. We found that mild vitamin A supplementation did not influence obesity, fat distribution, and glucose tolerance in males and females of the same genotype. In contrast, multiplex analysis of bioactive proteins in blood showed moderately increased concentrations (10-15%) of inflammatory IL-18 and MIP-1γ in vitA supplemented *vs*. control WT males. Marked decrease (28-31%) in concentrations of lymphotactin and tissue factor, a key protein contributing to thrombogenesis during injury, was achieved by vitA supplementation in WT females compared to control WT females. *Aldh1a1* deficiency reduced obesity, insulin resistance, suppressed many pro-inflammatory cytokines, and abolished the effects of vitA supplementation seen in WT mice. Our study revealed specific inflammatory and pro-thrombotic proteins in plasma regulated by dietary vitamin A and the critical role of endogenous vitA metabolism in these processes. The sex-specific decrease of plasma tissue factor concentrations by moderate dietary vitA supplementation could potentially reduce related pro-thrombotic states in obese females.

Keywords

Retinol; coagulation; sex differences; Raldh1; HOMA-IR index

INTRODUCTION

Metabolic syndrome (MSyn) is a cluster of conditions, including chronic inflammation, insulin resistance, obesity, dyslipidemia, hypertension, and pro-thrombotic state in the circulation that lead to type 2 diabetes, cardiovascular disease, and premature death [1].

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Obesity is one of the most prevalent MSyn conditions associated with development of type 2 diabetes and cardiovascular disease [2-5], because adipose tissue secretes an array of bioactive molecules contributing to chronic inflammation and a pro-coagulant state in blood [6]. There are two major types (depots) of white adipose tissue: visceral fat adjacent to vital organs and peripheral subcutaneous fat. Visceral fat produces cytokines, such as IL-6, TNFα, resistin, and retinol binding protein-4 (RBP-4), contributing to chronic inflammation and insulin resistance [7,8]. Subcutaneous fat, however, also produces anti-inflammatory adipokines, such as adiponectin, improving insulin sensitivity [7]. Sex is a major determinant of fat distribution and sexual dimorphism was also noted in blood concentrations of cytokines [8-10]. Both adipose depots secrete pro-thrombotic proteins, including tissue factor (TF) and PAI-1; however, PAI-1 is predominantly expressed in visceral fat [11]. Nonetheless, the shift of hemostasis (or haemostasis) to a pro-thrombotic state in Msyn is a sum of responses from different organs [12]. Liver, endothelial, and blood monocytes and platelets produce pro-thrombotic proteins, including factor VII, fibrinogen, and von Willebrand Factor (vWF) [12]. The rapid onset of MSyn in animal models without adipose tissue highlighted the important contribution of other tissues in the plasma pool of pro-inflammatory cytokines [13]. The pro-thrombotic state increases the risk for thrombosis, stroke, and heart attack in patients with MSyn [12]. The concentration of pro-coagulants and pro-inflammatory cytokines is increased in obese *vs.* lean patents and in animal models of obesity [14]. The pathways capable of improving the profile of cytokines and hemostasis in the blood of obese humans and rodents have not been sufficiently elucidated.

Dietary vitA (retinyl esters, retinol) serves as a precursor for retinaldehyde (Rald) and retinoic acid (RA) [15,16]. RA is a principal endogenous agonist for RA receptor (RAR) [17,18]. In addition, in many cell types, RA also regulates other transcription factors, including NF-κB, AP1, SP1, and STAT family [19-23]. These transcription factors also govern inflammation and production of pro-thrombotic proteins [24-26]. Notably, inflammation is associated with the depletion of vitA levels in plasma [27].

Intracellular RA is generated by the cytosolic aldehyde dehydrogenase-1 (ALDHa1, a2, and a3) enzyme family [16]. The enzymatic activity of ALDHa1 is critical for induction of fat-depot specific expression of *Zfp423, i.e.* 90% in visceral, and 70% in subcutaneous fat [28]. This transcription factor contributed to the higher expression of *Pparg* in subsutaneous than visceral fat [28]. PPARγ is a crucial regulator of adipogenesis, insulin sensitivity via adiponectin regulation, and suppressor of inflammation [29,30]. In *Aldh1a1*$^{-/-}$ mice, the diminished *Zfp423/ Pparg* expression rendered mice resistant to high-fat (HF) diet-induced obesity and glucose intolerance [28]. These effects were more pronounced in females than in males, because in males *Aldh1a2* and *Aldh1a3* compensated for RA deficiency [31]. However, it remains unknown how these metabolic changes influence inflammatory and hemostatic factors in blood of *Aldh1a1*$^{-/-}$ mice.

Consistent with its major role in obesity, vitA is stored in both white adipose depots [32]. In rodent genetic models of obesity and diabetes, vitA deficiency and vitA supplementation influence obesity, glucose tolerance, and inflammatory status [33-35]. However, due to the high capacity to store vitA in the hepatic stellate cells and white adipose tissue, dietary intake of vitA has only minimal impact on plasma concentrations. Previous studies showed that mice receiving diets containing different vitA concentrations (from 4.2 to 49 nmol/d) maintained the same plasma retinol concentrations (1.4–2.47 μmol/L) [36]. This physiological vitA levels in blood was sustained due to increased disposal rate (4.2–68.5 nmol/d) of vitA in the liver (1.2–11000 nmol) [36]. In humans, fortification with vitA of many basic products, such as milk, can lead to a moderate increase in vitA consumption (up to 5-fold recommended dietary allowance dose). It is unknown how a moderate increase in dietary levels of vitA influences Msyn conditions in obese mice.

In this study we showed that a moderate dietary vitA supplementation did not influence obesity and glucose tolerance in obese mice; however, it improved other MSyn conditions via suppression of pro-inflammatory and pro-thrombotic mediators in blood of obese female mice in *Aldh1a1*-dependent manner.

MATERIAL AND METHODS

Reagents

We purchased reagents from Sigma-Aldrich (St. Louis, MO) and cell culture media from Invitrogen (Carlsbad, CA) unless otherwise indicated.

Animal study was approved by the IACUC. *Aldh1a1* null mutant C57BL/6J mice (*Aldh1a1*$^{-/-}$) mice were developed by Dr. Gregg Duester and colleagues [16,37]. The metabolic characteristics of *Aldh1a1*$^{-/-}$ mice were described in [28,38]. Age- (8 weeks old) and sex-matched C57BL/6J (WT) and *Aldh1a1*$^{-/-}$ mice placed on a high fat diet (termed **HF group**, 45% kcal from fat with a standard 4IU vitA/g content or 20IU vitA (termed **HFA group**) purchased by Research Diets Inc., New Brunswick, NJ; HF: D12451 and HFA: D06031502). Each genetic/vitA group had 5 male and 5 female mice. Study lasted for 180 days. Metabolic cage measurements were performed after 120 days on HF and HFA diets. Food and water intake was measured after the mouse was acclimated to a powdered HF/HFA diets (4 days) in metabolic cages (Ancare, Charles River Laboratories).

Glucose tolerance test (GTT) and **insulin tolerance test** (ITT) was performed in overnight fasted mice. Animals were injected intraperitoneally with 0.004 mL 25% glucose/g body weight for GTT test and with insulin 0.1 U/ml, 0.005 ml/g body weight for ITT. HOMA-IR was calculated using the following formula: HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml) / 405 [39].

Multiplex bead-based platform

Blood was collected by submandibular puncture. Plasma proteins were analyzed using the multiplex bead based platform (Charles River biomarker services).

Enzyme-linked immunosorbent assay (ELISA)

Blood was collected by cardiac puncture. Mouse Adiponectin/Acrp30 Quantikine kit was purchased by R&D Systems (Minneapolis, MN). Plasma was diluted 25,000-fold with ELISA diluent prior to measurement. Hemolytic samples were omitted from the analysis. Adipose tissue (~150mg) were homogenized in RIPA buffer containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Hoffmann-La Roche Ltd, Indianapolis, IN). Protein concentration was determines using Pierce BCA protein kit (Thermo Scientific). The lysates containing 50μg protein/mL RIPA were analyzed using ELISA according to manufacturer's instructions.

Statistical analysis

Data are shown as mean±SD. Group comparisons were performed using unpaired Student *t*-test unless otherwise indicated.

RESULTS

Moderate increase in vitA in HF diet did not influence adipose tissue mass

The effect of moderate vitA supplementation (**HFA groups**) was studied in WT male and female mice on the HF diet (45% kcal from fat) (**HF groups**). Given that *Aldh1a1* is the

major RA-generating enzyme in white adipose tissue [28], we also examined the response to moderate vitA supplementation in *Aldh1a1*$^{-/-}$ mice. We defined as 'moderate' a five-fold increase in vitA levels compared to a standard 4IU/g vita content in HF. We selected male and female mice of similar weight at the beginning of the study (Fig. 1a, b). The enhanced vitA content in the HF diet did not influence weight gain in any of the studied groups. WT male and female mice gained more weight than *Aldh1a1*$^{-/-}$ mice; however, this gain was identical in HF and HFA-fed mouse groups. Similarly, food and water intake, measured in metabolic cages was not statistically different between HF and HFA fed mice in each sex and genotype group (Fig. 1c, d). The white adipose tissue mass in both subcutaneous and visceral fat depots was markedly different between WT and *Aldh1a1*$^{-/-}$ sex-matched groups (Fig. 2a, b), in agreement with our previous reports [31]. However, in each sex and genotype groups, moderate vitA supplementation per se did not influence the adipose tissue mass (Fig. 2a, b). Finally, neither liver weight (data not shown) nor liver to weight ratio (Fig. 2c) was changed in response to moderate vitA supplementation. White adipose and hepatic tissue are the major storage sites for dietary vitA and produce a variety of cytokines, adipokines, and hepatokines that regulate glucose metabolism [14,32]. Therefore, we investigated glucose metabolism in HF and HFA fed mouse groups.

Moderate increase in vitA in HF diet improves glucose metabolism in obese males

Moderate vitA supplementation had no effect on the ITT, GTT, insulin concentration, or HOMA-IR index in WT male and female mice (Fig. 3). Both *Aldh1a1*$^{-/-}$ male and female mice had improved ITT, while GTT was only decreased in *Aldh1a1*$^{-/-}$ females compared to sex-matched WT groups (Fig. 3a, b). Both GTT and ITT responses were significantly improved in *Aldh1a1*$^{-/-}$ males supplemented with vitA compared to a control HF-fed *Aldh1a1*$^{-/-}$ male group (Fig. 3a, b, HFA *vs*. HF). The blood insulin levels and HOMA-IR index was decreased in *Aldh1a1*$^{-/-}$ *vs*. WT males (Fig. 3c, d) Dietary vitA had no effect on blood insulin levels and HOMA-IR index in any of the studied groups. Similarly, insulin-sensitizing adipokines such as adiponectin levels in circulation were also significantly changed between sex-matched WT and *Aldh1a1*$^{-/-}$ mice (Fig. 4a); however, vitamin A supplementation did not influence adiponectin levels in studied groups. Adiponectin concentrations in homogenized visceral (retroperitoneal) and subcutaneous fat pad were also not changed in HF *vs*. HFA sex-matched groups (Fig. 4b, c). We concluded that HF diet-mediated metabolic changes were more dependent on the metabolizing ALDH1a1 enzyme than on the moderate increase in vitA content in HF diet. Although adipose tissue is an important contributor to development of Msyn, some Msyn conditions, such as chronic inflammation also emerges in the absence of white adipose tissue [13] due to the increased secretion of pro-inflammatory mediators in blood by other tissues, including hepatic, intestinal, vascular, hematopoietic, and immune, which can affect immune status and hemostasis in blood [24-26]. Therefore, we tested concentrations of pro-inflammatory and pro-thrombotic proteins in blood.

Increased content of vitA in diet decreases pro-thrombotic factors in obese females

We measured several metabolites in a multiplex format in the blood of WT and *Aldh1a1*$^{-/-}$ males and females fed HF and HFA diets (Table 1, 2). We found that *Aldh1a1* deficiency significantly altered the profile of pro-inflammatory cytokines and pro-thrombotic factors in blood. We detected marked (≥2-fold) decrease in leptin, MCP-1, MCP-3, CD40, CD40L, IP-10, and IL-10 in *Aldh1a1*$^{-/-}$ compared to WT sex-matched mice on the HF diet. The decrease in MIP-1γ, MIP-1, MIP-3, IL1β, IL-5, and IL-18, was less pronounced but also significant in *Aldh1a1*$^{-/-}$ *vs*. WT sex-matched mice on the HF diet. The example of this regulation pattern is shown for leptin in Fig. 5a.

Dietary vitA supplementation resulted in a sex-specific regulation of only a few cytokines. Lymphotactin was reduced (-28%) in WT female mice on HFA *vs*. HF diet, but was maintained at the same levels in WT males (Fig. 5b). *Aldh1a1* deficiency only led to a significant reduction of lymphotactin levels in the female group.

In WT males, vitA supplementation moderately increased blood concentration of two cytokines IL-18 and MIP-1γ (Fig. 5c, d). This effect was abolished in *Aldh1a1*$^{-/-}$ males.

An unexpected finding in our study was the sex-specific effect of both, vitA supplementation and *Aldh1a1* deficiency on the blood hemostatic factors. Supplementation with vitA decreased tissue factor (-31%) in female, but not in male (+24%) WT mice on HFA *vs*. HF diet (100%). *Aldh1a1* deficiency reduced tissue factor levels in both male and female *Aldh1a1*$^{-/-}$ *vs*. WT mice (Fig. 5e). Factor VII and thrombopoietin were significantly reduced in female *Aldh1a1*$^{-/-}$ *vs*. WT mice on HF and/or HFA diet; in males these factors were only significantly decreased on the HFA diet (Table 1, 2). Strikingly, vWF was elevated in the vitA supplemented group only in *Aldh1a1*$^{-/-}$ male mice, while females maintained similar vWF blood levels in all groups. Thus, levels of pro-thrombotic and pro-inflammatory proteins were improved by vitA in female but not in male WT obese mice.

Consistent with its expression in adipose tissue, leptin was correlated with the mass of white fat comprising of retroperitoneal visceral and subcutaneous fat pads (Fig. 6a). Pro-inflammatory cytokines such as MCP-1, IP-10 (Fig. 6b, c), which are expressed in adipose tissues, also significantly correlated with white fat mass, in contrast to lymphotactin (Fig. 6d). Pro-thrombotic tissue factor is expressed in adipose and predominantly in vascular cells [11]. Correspondingly, tissue factor showed a weaker, non-significant correlation with white adipose tissue mass (Fig. 6e), whereas vWF factors, produced by hepatic cells, showed no association with adipose tissue (Fig. 6f). Thus, both lymphotactin and tissue factor, that were altered in response to vitA dietary supplementation in females, were not correlated with the mass of major white adipose tissue depots.

DISCUSSION

Fortification of dairy products with vitA can potentially lead to a moderate increase in vitA consumption. In light of controversial reports on the obesogenic and anti-obesogenic effects of vitA consumption, treatments with RA, and genetic deficiency in vitamin A-metabolizing enzymes in different rodent models or patients [19,33,34,40-42], it is important to understand metabolic effects of moderate vitA supplementation. Whereas obesity is an important condition, Msyn can also emerge without obesity [13]; therefore, we also assessed how dietary vitA supplementation influences other critical Msyn manifestations: such as plasma levels of mediators of inflammation and thrombosis. We found that moderate vitA increase in diet only influenced specific cytokines (MIF-1γ, IL-18, lymphotactin) and one pro-thrombotic factor (tissue factor) in a sex-specific manner (Fig. 7). These dietary effects appeared to be independent of adipose tissue mass (Fig.6, 7). However, were abolished in *Aldh1a1*$^{-/-}$ mice, suggesting the dependence of dietary vitA effects on endogenous metabolism by ALDH1a1. The overall role of vitA metabolism utilizing endogenous stores was more profound: *Aldh1a1*$^{-/-}$ mice were protected from obesity, glucose tolerance, liver steatosis [38], and had decreased levels of pro-inflammatory and pro-thrombotic mediators in blood (Fig.7). The obesity in mice appeared to be a major determinant of their cytokine profile as suggested by correlation analysis (Fig. 6). The plasma levels of MCP-1, IP-10, and other pro-inflammatory cytokines known to be secreted from adipose tissue [43-45], were reduced in *Aldh1a1*$^{-/-}$ mice proportionally to their adipose tissue mass. We proposed two principal modes of vitA action in inflammation and hemostasis (Fig. 7): 1) Enzyme-controlled utilization of endogenous vitA for regulation of cytokines in peripheral tissues,

such as adipose, hepatic and other tissues, and 2) Direct responses to postprandial vitA concentrations by vascular and immune cells.

Based on the well-documented links between vitA/inflammation and vitA/obesity [19-23,33,34,40-42], we initially hypothesized that inflammatory and hemostatic factors in the blood of WT and *Aldh1a1*$^{-/-}$ mice on the HF and HFA diet will change proportionally to the level of obesity and occur in a sex-specific manner. This indirect adipocentric mechanism is based on the fact that adipose tissue mass is markedly lower in *Aldh1a1*$^{-/-}$ compared to WT mice.

Hypertrophic adipocytes and macrophages in adipose tissue express leptin, MCP-1, MCP-3, CD40, CD40L, IP-10 IL-10, MIP-1γ, MIP-1, MIP-3, IL-18, IL1b, IL-5, and tissue factor [43-45]. We found that moderate vitA supplementation did not alter weight gain, fat formation, or fat distribution induced by HF diet in WT or *Aldh1a1*$^{-/-}$ mice (Fig. 1, 2). This finding was in consonance with previous reports [46]. The glucose metabolism and insulin-sensitizing adiponectin also was not altered in response to the moderate change in vitA content in the HF diet in these mice (Fig. 3, 4). In contrast, *Aldh1a1* deficiency prevented development of obesity and glucose tolerance on both HF and HFA diets (Fig. 1-3), ref. [28,31,38]. Both liver and adipose tissues can effectively store high concentrations of vitA that could eliminate the impact of the different dietary levels of vitA in these peripheral tissues.

Consistent with the known contribution of adipose tissue to the pool of adipokines, pro-inflammatory cytokines, and pro-thrombotic factors in blood [14,24-26], leptin, MCP-1, MCP-3, CD40, CD40L, IP-10, IL-10, MIP-1γ, MIP-1, MIP-3, IL-18, IL1β, IL-5, and tissue factor were reduced in lean *Aldh1a1*$^{-/-}$ compared to obese WT mice. Significant correlation between white adipose tissue mass and leptin, MCP-1, and IP-10 suggested the pivotal role of adipose tissue in the production of these cytokines (Fig. 6). The lack of correlation between adipose tissue and tissue factor or pro-thrombotic factors VII and vWF (Fig. 6) is in agreement with the known production of these proteins by vascular and hepatic tissues, respectively. Hyperlipidemia and non-alcoholic liver steatosis is associated in some studies with higher production of factors VII and vWF [47-50]. *Aldh1a1*$^{-/-}$ mice resist HF-diet induced hyperlipidemia and triglyceride accumulation in the liver seen in WT mice on an obesogenic diet [38]. It is plausible that decreased plasma levels of factors VII and vWF are due to the differences in hepatic lipid metabolism in WT and *Aldh1a1*$^{-/-}$ mice. Specific mechanisms regulating cytokine regulation by ALDH1a1 and the role of endogenous vitA metabolism were beyond the scope of this study identifying ALDH1a1-sensitive pool of bioactive plasma proteins. Regardless of the mechanism, our study demonstrates that ALDH1a1, possibly via endogenous vitA utilization, controls the majority of metabolic pathways and mediators of inflammation and hemostasis in the blood.

Aldh1a1 is the major enzyme in human and mouse white adipose tissue capable of RA production [28]. In adipocytes, sex-specific differences are mediated in part by estrogen, which suppresses expression of Aldh1a2 and Aldh1a3 enzymes [31], therefore, *Aldh1a1*$^{-/-}$ females have less visceral adipose tissue than *Aldh1a1*$^{-/-}$ males. In consonance with the adipose reduction pattern, the blood levels of leptin were lower in *Aldh1a1*$^{-/-}$ females than males (Fig. 5a right *vs*. left panel). Other cytokines and pro-thrombotic factors circulated at similar levels in males and females. Notably, all HFA-diet-dependent changes occurred in a sex-specific fashion. While WT males had increased plasma concentrations of MIP-1γ and IL-18, WT females had reduced lymphotactin and tissue factor concentrations on HFA compared to HF diet (Fig. 5). In both sexes, the effect of dietary vitamin A was abolished in *Aldh1a1*$^{-/-}$ mice suggesting that the metabolic conversion to RA contributed to these effects. Altered concentrations of MIP-1γ and IL-18 in males and in tissue factor and

lymphotactin in females were independent of changes in adipose tissue mass, and could be produced by another, *e.g.* immune and vascular tissue. Dietary vitA is assembled in enterocytes into chylomicrons and transported into the lymphatic system for uptake in peripheral tissues (Fig. 7) [15]. Lymphotactin is expressed in intestinal and immune cells [51], whereas vascular cells express tissue factor [11]. These cell types are exposed to dietary and postprandial vitA that could regulate synthesis of lymphotactin and tissue factor in response to moderate changes of dietary vitA. A potential mechanism leading to the sex-specific levels of these cytokines in blood may include ALDH1 enzymes, because all these effects were abolished in *Aldh1a1* deficient males and females (Fig. 5). ALDH1 family of enzymes also plays an important role in other tissues, notably immune cells, including dendritic cells and macrophages [20,52]. Due to estrogen suppression of *Aldh1a2* and *Aldh1a3* expression [31], cells with low *Aldh1a1* expression or activity have diminished RA production [28]. RA inhibits tissue factor expression in endothelial and leukemic cell cultures, probably via RAR/RXR dependent mechanisms [53,54]. However, RA also directly regulates expression of RAR receptors [55] and required for the differentiation of lymphatic vasculature [56], that expect to be impaired in $Aldh1a1^{-/-}$ females. Numerous studies have documented a role of estrogen in the regulation of coagulation cascade [57]. Ethynyl estradiol improved the pro-thrombotic phenotype in obese rats [57]. We can speculate that estrogen- and vitamin A-activated pathways synergize their action; however, future studies need to identify cells contributing to sex-specific production of tissue factor, and MIP-1γ, IL-18, lymphotactin and dissect mechanisms underlying vitA action.

The major finding in our study is that a moderate increase of vitA in the HF diet improved pro-thrombotic tissue factor levels in obese female WT mice, while in obese males elevated vitA content in the HF diet was associated with a moderate increase in inflammation.

Obese men and women showed sexual dimorphism in the expression of Aldh1 enzymes [31]. The sex-specific response to moderate vitA supplementation in obese mice may suggest that dietary vitA can also exert different metabolic effects in obese men and women. In obese male mice, HFA diet moderately (up to 15%) increased levels of pro-inflammatory MIP-1γ and IL-18 cytokines. IL-18 is an integral part of inflammasome secreted by adipose tissue [43], which is also causally implicated in the development of obesity [58]. In female mice, moderately-elevated dietary vitA content resulted in a profound (-30%) decrease in tissue factor levels in blood (Fig. 5e) suggesting a reduced propensity to pro-thrombotic events. Lymphotactin was found in human subcutaneous fat in association with obesity [59]. In our study, this cytokine was selectively and effectively reduced in obese females on HFA diet (Fig. 5b). More studies are needed to establish the function of this cytokine in obesity. VitA regulated numerous signaling and transcriptional pathways [19]. Our study demonstrated an array of inflammatory and pro-thrombotic factors that are dependent on dietary vitA and/or endogenous vitA metabolism. Dietary vitA can potentially provide an opportunity to regulate blood levels of cytokines and hemostatic factors for therapeutic purposes.

CONCLUSIONS

A moderate increase in vitA in the HF diet did not influence obesity and glucose sensitivity. It regulated adipokines, inflammatory cytokines, and hemostatic factors in a sex-specific and *Aldh1a1*-dependent fashion. VitA in the diet increased concentrations of pro-inflammatory cytokines in males, whereas in females it reduced lymphotactin and improved the hemostatic blood profile by diminished production of tissue factor. Our data demonstrate that vitA in the diet can differentially impact males and females and provide evidence of improved hemostatic factors in obese females.

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Abbreviations

Aldh1a1	Aldehyde dehydrogenase 1 family, member a1
***Aldh1a1*$^{-/-}$**	Knockout in *Aldh1a1*
Aldh1a2	Aldehyde dehydrogenase 1 family, member a2
Aldh1a3	Aldehyde dehydrogenase 1 family, member a3
HF	high fat diet (4IU vitamin A/g)
HFA	high fat vitamin A diet (20 IU vitamin A/g)
GTT	Glucose tolerance test
AP1	Activator Protein 1
CC	CCL Chemokine Group of Cytokines
CRP	C Reactive Protein
CXC	CXCL Chemokine Group of Cytokines
HOMA-IR	Homeostasis Model of Assessment of Insulin Resistance
IL-18	Interleukin-18
IL-10	Interleukin-10
IL-1β	Interleukin-1 beta
IL-5	Interleukin-5
IP-10	Interferon gamma-induced protein 10
ITT	Insulin tolerance test
LIF	Leukemia Inhibitory Factor

MCP-1	Monocyte Chemoattractant Protein-1
MCP3	Monocyte Chemoattractant Protein-3
MCP-5	Monocyte Chemoattractant Protein-5
MDC	Macrophage-Derived Chemokine
MIP-1α	Macrophage Inflammatory Protein-1 alpha
MIP-3β	Macrophage Inflammatory Protein-3 beta
MIP-1γ	Macrophage Inflammatory Protein-1 gamma (alias CCL9)
MIP-2	Macrophage Inflammatory Protein-2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	Plasminogen Activator Inhibitor-1
PPARγ	Peroxisome Proliferator-activated Receptor gamma
PPARβ/δ	Peroxisome Proliferator-activated Receptor delta
RAR	Retinoic Acid Receptor
Rald	Retinaldehyde
RA	Retinoic acid
RDA	Recommended Dietary Allowance
RBP	Retinol Binding Protein
SP1	Specificity Protein 1
STAT	Signal Transducer and Activator of Transcription
TF	Tissue Factor
TIMT-1	Tissue Inhibitor of Metalloproteinase Type-1
VWF	von Willebrand Factor
ZFP423	Zinc-Finger Protein Transcription Factor 423

HIGHLIGHTS

- A 5-fold increase in vitamin A in a high-fat diet (HFA vs. HF diet) does not influence obesity in mice
- HFA *vs*. HF diet increased concentrations of pro-inflammatory cytokines MIP-1 and IL-18 in males
- HFA *vs*. HF diet reduced lymphotactin and pro-thrombotic tissue factor in female mice
- Dietary Vitamin A does not influence glucose tolerance in obese mice
- Effects of dietary vitamin A depend on Aldh1a1

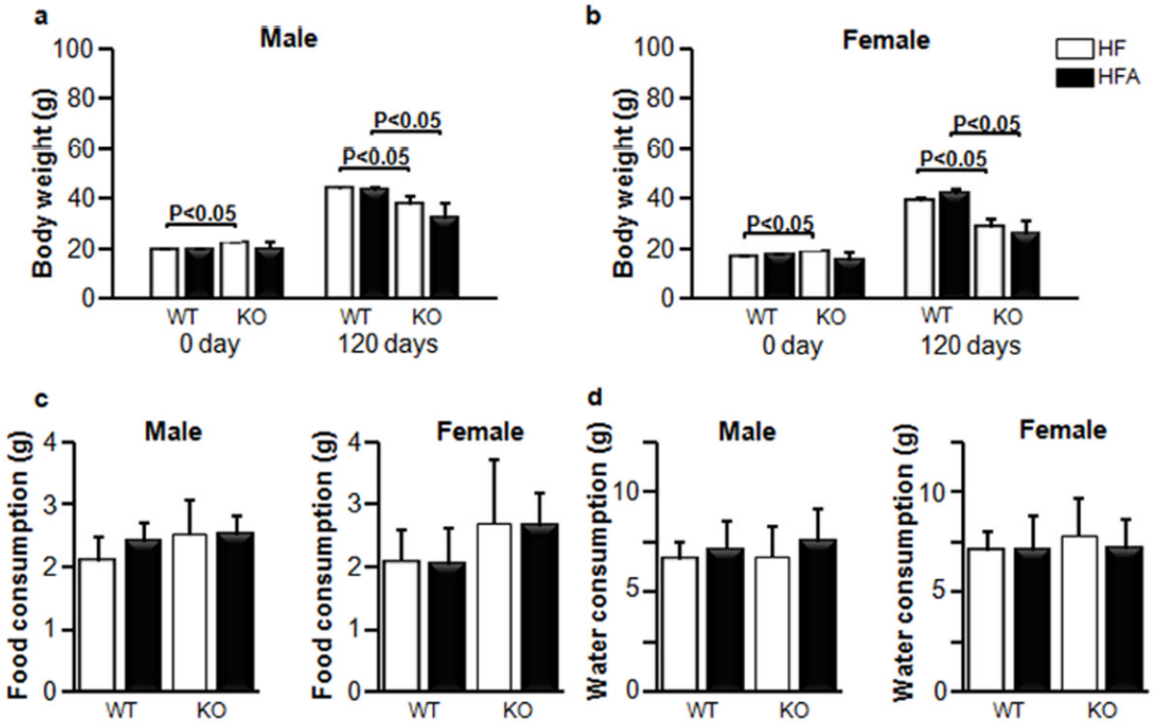

Figure 1. High-fat (HF) and high-fat with moderately elevated vitA content (HFA) have similar responses on whole body weights and food and water consumptions in WT and in *Aldh1a1*$^{-/-}$ (KO) mice

Whole body weights are shown before and after 120 days diet treatment for male (**a**) and female (**b**) mice (n=5 for all mouse groups). Effect of HF and HFA diets on food (**c**) and water (**d**) consumptions for male and female groups. Food and water consumption was measured in metabolic cages (n=4 for all mouse groups). Data are shown as mean±SD. The *p*-values were calculated by Student's t-test.

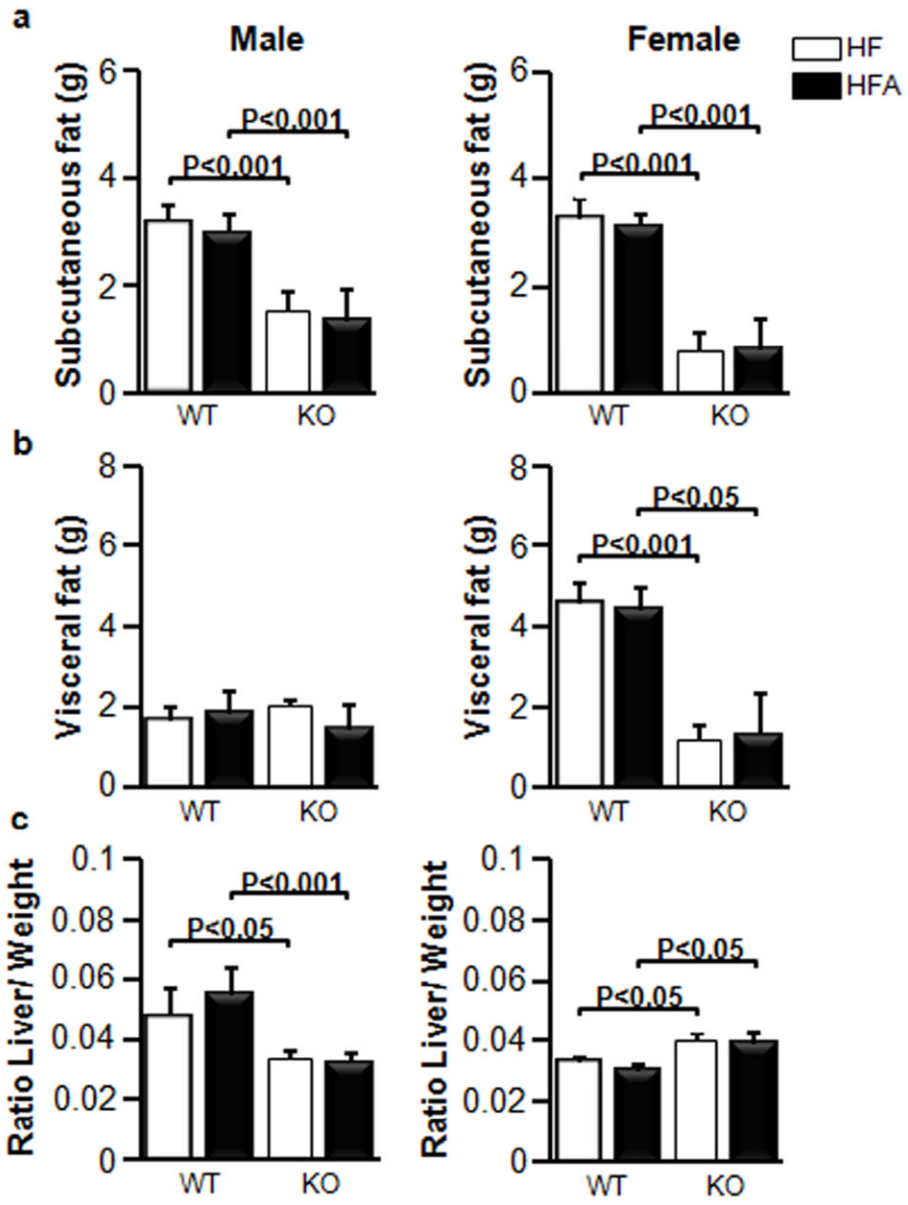

Figure 2. Similar mass of white adipose tissue depots in WT and KO mice fed HF and HFA diets

Mice (n=5 for all mouse groups, same as Fig.1) were fed HF and HFA diets for 180 days, then major adipose (**a**) subcutaneous, (**b**) visceral fat weights, and (**c**) the ratio of liver mass/ body weight (relative liver weight) in WT and in KO mice. Data are shown as mean±SD. The *p*-values were calculated by Student's t-test (n=5 for all mouse groups).

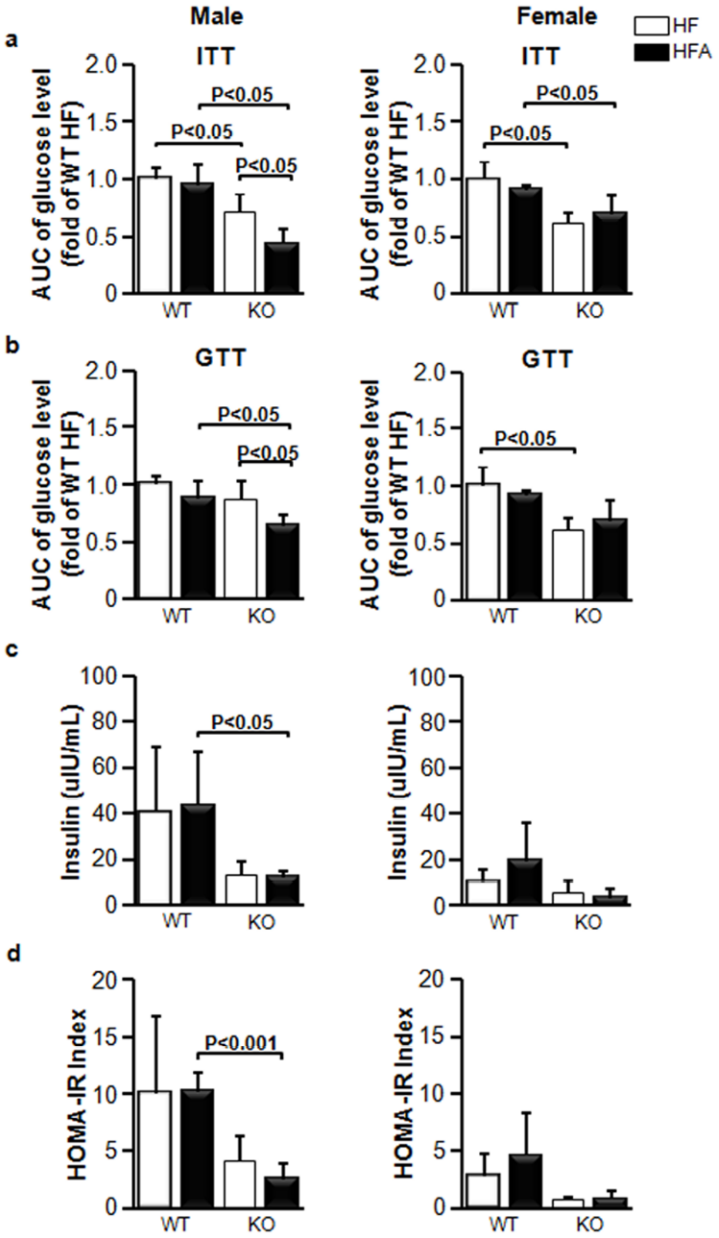

Figure 3. Similar glucose tolerance in WT and KO mice fed HF and HFA diets

Comparison of areas under the curve (AUC) for (**a**) insulin tolerance tests (ITT) and (**b**) glucose tolerance tests (GTT) in WT and in KO male and female mice after 120 days on HF and HFA diets. (**c**) Fasting blood insulin levels in the mice after 120 days of treatment on HF and HFA diets. (**e**) Homeostasis Model of Assessment of Insulin Resistance (HOMA-IR) for the mice after 120 days of treatment on HF and HFA diets. Data are shown as mean±SD. The *p*-values were calculated by Student's t-test. (n=4 for all mouse groups for ITT and GTT tests; n=5 for insulin measurements).

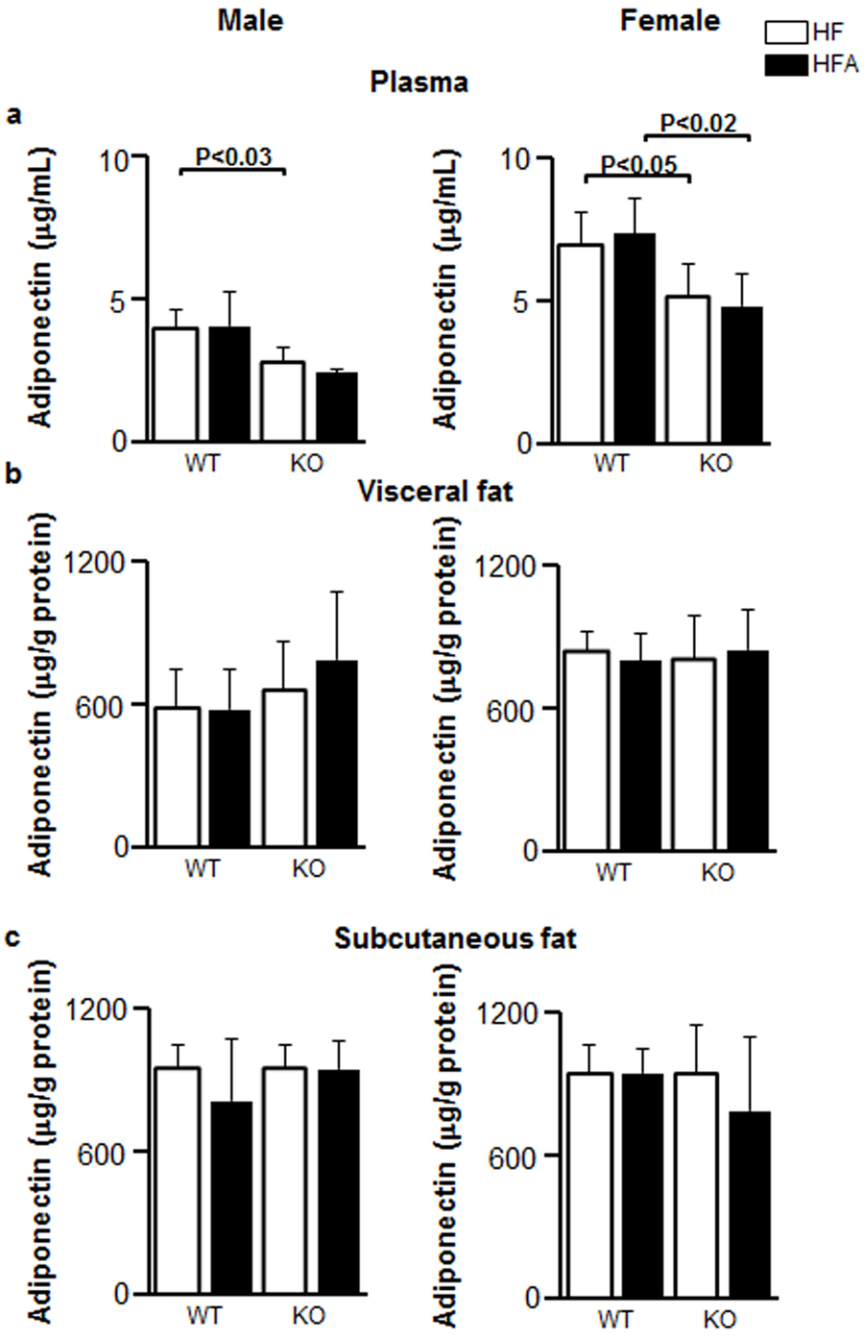

Figure 4. Similar adiponectin levels in blood, visceral, and subcutaneous tissues in WT and KO mice fed HF and HFA diets

The concentrations of adiponectin in plasma (**a**), and homogenates of visceral (**b**) and subcutaneous (**c**) adipose tissues in response to HF and HFA diets in WT and KO male and female mice. Adiponectin concentrations were measured by ELISA. Tissues homogenates were normalized by protein content to 50 µg protein/mL prior to ELISA. Data are shown as mean±SD. The *p*-values were calculated by Student's t-test. (n=5 for all mouse groups).

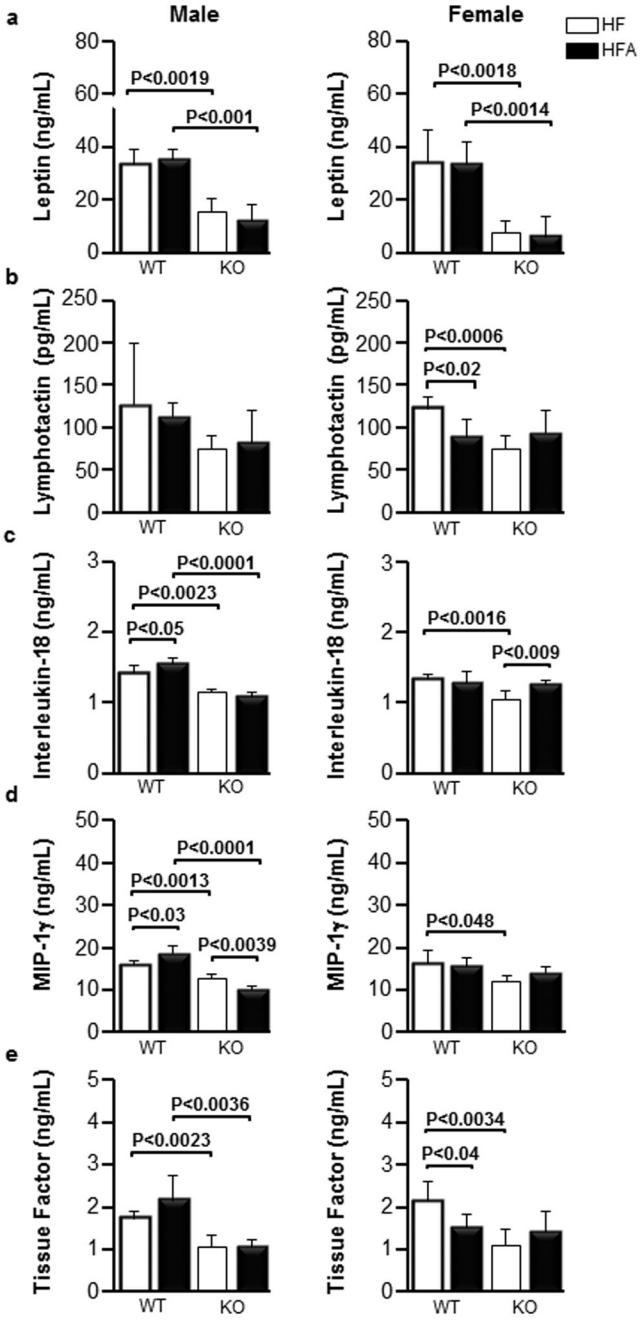

Figure 5. HFA selectively influence blood concentrations of cytokines and tissue factor in males and females in *Aldh1a1*$^{-/-}$-dependent manner

The concentrations of leptin (**a**), circulating lymphotactin (**b**), MIP-1γ (**c**), IL-18 (**d**), and tissue factor (**e**) in the blood in response to HF and HFA diets in WT and KO male and female mice. Data are shown as mean±SD. The *p*-values were calculated by Student's t-test. WT (n=5 for all mouse groups, same as Fig. 1).

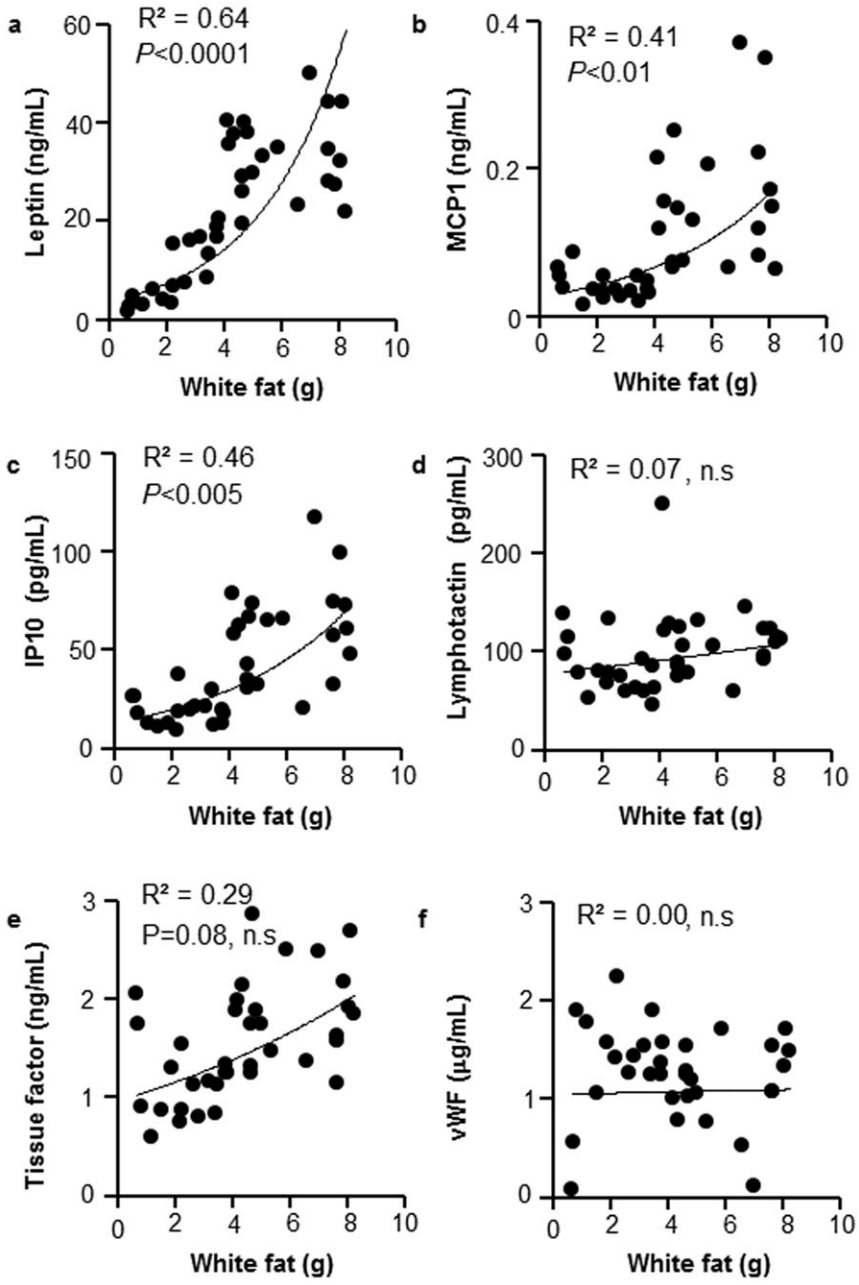

Figure 6. White adipose tissues mass correlate with plasma concentration of adipokines and cytokines but not pro-thrombotic factors

The white adipose tissue mass is a sum of weights of two visceral (retroperitoneal) and two subcutaneous fat pads. Concentrations of cytokines are the same as in Fig. 5. Data include all groups of WT and *Aldh1a1*$^{-/-}$ mice on HF and HFA diets. Correlations between white adipose tissue mass and leptin (**a**), MCP-1 (**b**), IP-10 (**c**), lymphotactin (**d**), tissue factor (**e**), and VWF (**f**). n=37, Pearson correlation test.

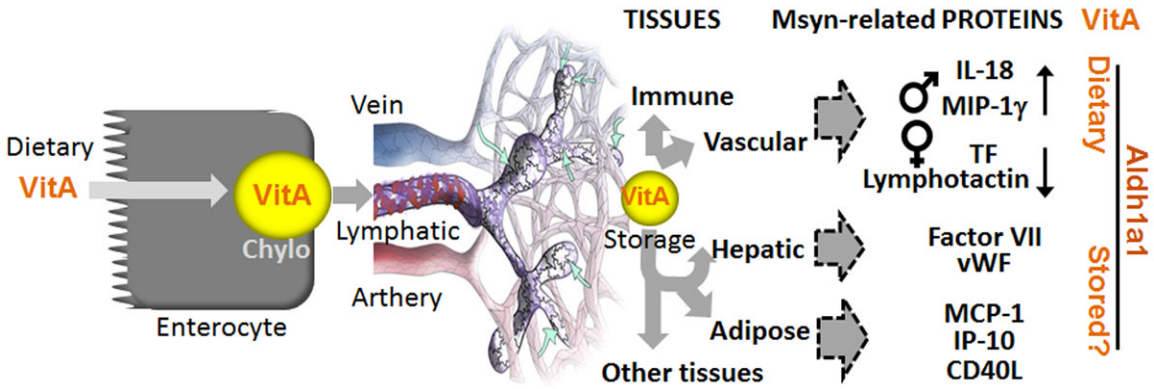

Figure 7. Hypothetical regulatory mechanism for pro-inflammatory cytokines and pro-thrombotic factors by dietary and endogenous vitA

Dietary vitA is assembled into chylomicrons in enterocytes and transported in lymphatic system for the uptake in peripheral tissues. Intestinal, immune and vascular cells interact with vitA-loaden chylomicrons and respond to changes in dietary concentrations. Lymphotactin is expressed in intestinal and immune cells, whereas and vascular cells express tissue factor (TF) in females. In males, dietary vitA mildly increases IL-18 and MIP-1γ. The remaining vitA in chylomicron remnants is stored in adipose and hepatic tissues. In these tissues Aldh1a1, together with other enzymes, participate in the metabolism of endogenous vitA. Consequently, Aldh1a1 deficiency influences many adipokines, cytokines and hepatic pro-thrombotic factors. Circulating and resident in peripheral tissues immune cells may contribute to blood concentrations of pro-inflammatory cytokines.

Table 1

Effect of moderate vitamin A supplementation on the inflammatory and haemostatic factors in blood of male mice

Mice were fed a high-fat diet (HF, 4IU vitamin A/g chow) and same diet with increased vitamin A content (HFA, 20IU vitamin A/g chow) for 180 days. Blood was collected after 120 d on the diet. Data are shown as mean±SD, n=5 per each group. P, Mann-Whitney *U* test.

	WT male		P value[1]	KO male		P value[2]	P value[3]	P value[4]
	HF	HFA		HF	HFA			
Leptin (ng/mL)	33.3±5.9	35.22±3.9	N/S	15.3±5.1	11.83±6.2	N/S	0.0019	0.0001
CRP (μg/mL)	0.12±0.03	0.13±0.06	N/S	0.16±0.03	0.14±0.03	N/S	N/S	N/S
CCL Chemokine								
MIP-1γ (ng/mL)	16.1±0.6	18.5±1.7	0.0311	12.65±1.0	10.07±0.8	0.0039	0.0013	0.0001
MCP-1 (pg/mL)	126±59	158±71.3	N/S	38.6±12.5	28.8±8.5	N/S	0.0233	0.0038
MCP-3 (pg/mL)	254±106	279±76	N/S	104±26	100±35	N/S	0.0297	0.0014
MCP-5 (pg/mL)	25.6±11.4	32.7±14.8	N/S	16.7±3.3	14.9±6.6	N/S	N/S	N/S
MDC (pg/mL)	346±97	385±119	N/S	206 ±61	213±30	N/S	0.0429	N/S
MIP-1α (ng/mL)	0.5±0.1	0.5±0.1	N/S	0.4±0.01	0.34±0.03	N/S	0.0192	0.0001
MIP-3β (ng/mL)	0.3±0.1	0.3±0.1	N/S	0.3±0.1	0.3±0.1	N/S	N/S	N/S
Eotaxin (μg/mL)	0.9±0.4	0.9±0.3	N/S	0.5±0.1	0.4±0.1	N/S	N/S	0.0106
CXCL Chemokine								
Lymphotactin (pg/mL)	127±72	113±17	N/S	74.4±16.3	82.1±38.1	N/S	N/S	N/S
MIP-2 (pg/mL)	20.3±10.7	19.9±12.5	N/S	3.7±2.7	4.7±1.8	N/S	0.0203	0.0272
IP-10 (pg/mL)	56.3±21.9	58.8±10.0	N/S	21.7±5.4	19.7±10.2	N/S	0.0185	0.0003
TNF Family Proteins								
CD40 (pg/mL)	141±20	162±25	N/S	65±14	47±10	N/S	0.0003	0.0001
CD40 Ligand (pg/mL)	497±102	474±98	N/S	212±65	140±68	N/S	0.0020	0.0002
Haemostatic Factors								
TF (ng/mL)	1.7±0.2	2.2±0.6	N/S	1.1±0.3	1.1±0.2	N/S	0.0023	0.0036
Endothelin-1 (pg/mL)	39.3±6.3	41.8±7.9	N/S	33.9±6.5	29.6±5.3	N/S	N/S	0.0209
Factor VII (ng/mL)	1.1±0.21	1.2±0.15	N/S	0.7±0.2	0.5±0.1	N/S	0.0197	0.0001
Thrombopoietin (ng/mL)	10.8±1.3	10.2±0.7	N/S	7.5±1.0	7.9±0.5	N/S	0.0040	0.0003
vWF (ng/mL)	1056±209	1203±389	N/S	1393±138	1750±384	N/S	0.0365	N/S

	WT male		P value[1]	KO male		P value[2]	P value[3]	P value[4]
	HF	HFA		HF	HFA			
TIMT-1 (ng/mL)	1.4±0.3	1.7±0.2	N/S	0.8±0.04	0.8±0.3	N/S	0.0098	0.0004
Interleukin Inflammatory Proteins								
IL-18 (ng/mL)	1.4±0.1	1.6±0.1	0.05	1.2±0.04	1.1±0.1	N/S	0.0023	0.0001
IL-10 (pg/mL)	295±84	328±100	N/S	166±34	162±36	N/S	0.0242	0.0083
IL-1β (ng/mL)	1.7±0.2	1.7±0.2	N/S	1.2±0.2	1.1±0.1	N/S	0.0130	0.0003
NIL-5 (ng/mL)	0.3±0.1	0.3±0.04	N/S	0.2±0.04	0.2±0.02	N/S	0.0243	0.0006

[1] P value, difference in HF vs. HFA chow for WT male mice

[2] P value, difference in HF vs. HFA chow for KO male mice

[3] P value, difference in WT vs. KO male mice for HF chow

[4] P value, difference in WT vs. KO male mice for HFA chow

N/S – samples are not significantly different

Table 2

Effect of moderate vitamin A supplementation on the inflammatory and haemostatic factors in blood of female mice

Mice were fed a high-fat diet (HF, 4IU vitamin A/g chow) and same diet with increased vitamin A content (HFA, 20IU vitamin A/g chow) for 180 days. Blood was collected after 120d on the diet. Data are shown as mean±SD, n=5 per each group. P, Mann-Whitney *U* test.

	WT female		P value[1]	KO female		P value[2]	P value[3]	P value[4]
	HF	HFA		HF	HFA			
Leptin (ng/mL)	33.9±12.1	33.2±8.6	N/S	7.2±4.9	5.9±7.5	N/S	0.0018	0.0014
CRP (μg/mL)	0.11±0.05	0.17±0.03	0.0317*	0.13±0.0	0.12±0.05	N/S	N/S	N/S
CCL Chemokine								
MIP-1γ (ng/mL)	16.2±3.2	15.6±1.8	N/S	11.9±1.3	13.9±1.4	N/S	0.0483	N/S
MCP-1 (pg/mL)	229±131	108±47	N/S	44.4±26.0	49.8±15.9	N/S	0.0146	0.0348
MCP-3 (pg/mL)	427±298	318±95	N/S	134±43	172±43	N/S	N/S	0.0171
MCP-5 (pg/mL)	36±15	41±20	N/S	16±5	215±10	N/S	0.0250	N/S
MDC (pg/mL)	444±207	267±55	N/S	241±63	251±70	N/S	N/S	N/S
MIP-1α (ng/mL)	0.46±0.07	0.40±0.03	N/S	0.37±0.04	0.40±0.05	N/S	0.0425	N/S
MIP-3β (ng/mL)	0.35±0.03	0.31±0.08	N/S	0.24±0.03	0.25±0.05	N/S	0.0007	N/S
Eotaxin (μg/mL)	1.8±1.2	1.2±0.8	N/S	0.8±0.5	0.8±0.7	N/S	N/S	N/S
CXCL Chemokine								
Lymphotactin (pg/mL)	123±13	88±21	0.0197	75±15	93±27	N/S	0.0006	N/S
MIP-2 (pg/mL)	42.8±44.5	11.7±6.9	N/S	6.1±2.8	6.2±5.4	N/S	N/S	N/S
IP-10 (pg/mL)	75.9±30.2	49.3±27.6	N/S	14.8±3.8	20.7±9.7	N/S	0.0020	N/S
TNF Family Proteins								
CD40 (pg/mL)	116.8±20.6	98.2±21.2	N/S	50.2±10.3	57.6±15.6	N/S	0.0002	0.0127
CD40 Ligand (pg/mL)	585±66	516±103	N/S	217±49	242±130	N/S	0.0001	0.0109
Haemostatic Factors								
TF (ng/mL)	2.15±0.43	1.49±0.33	0.0405	1.09±0.38	1.41±0.05	N/S	0.0034	N/S
Endothelin-1 (pg/mL)	46.5±3.9	38.6±8.8	N/S	37.7±7.2	29.0±4.9	N/S	0.0443	N/S
Factor VII (ng/mL)	1.3±0.2	1.1±0.15	N/S	0.7±021	0.8±0.15	N/S	0.0037	0.0118
Thrombopoietin (ng/mL)	10.6±1.4	9.6±1.5	N/S	8.3±1.6	8.4±1.9	N/S	0.0439	N/S
vWF (ng/mL)	1082±707	1102±438	N/S	1087±620	975±636	N/S	N/S	N/S

	WT female		P value[1]	KO female		P value[2]	P value[3]	P value[4]
	HF	HFA		HF	HFA			
TIMT-1 (ng/mL)	1.3±0.3	1.2±0.1	N/S	0.9±0.5	1.0±0.4	N/S	N/S	N/S
Interleukin Inflammatory Proteins								
IL-18 (ng/mL)	1.3±0.1	1.3±0.2	N/S	1.0±0.1	1.3±0.1	0.0090	0.0016	N/S
IL-10 (pg/mL)	347±99	439±299	N/S	154±39	179±91	N/S	0.0036	N/S
IL-1β (ng/mL)	1.6±0.1	1.6±0.3	N/S	1.3±0.1	1.4±0.3	N/S	0.0018	N/S
IL-5 (ng/mL)	0.4±0.04	0.3±0.1	N/S	0.3±0.1	0.3±0.1	N/S	0.0443	N/S

[1] P value, difference in HF vs. HFA chow for WT female mice

[2] P value, difference in HF vs. HFA chow for KO female mice

[3] P value, difference in WT vs. KO female mice for HF chow

[4] P value, difference in WT vs. KO female mice for HFA chow

N/S – samples are not significantly different

[*] P value was calculated using two-tailed U Test (Mann-Whitney U Test)