Effects of Dietary Calcium on Body Composition and Lipid Metabolism in Rats

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the degree of

MASTER of SCIENCE

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Contents

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Abstract

Calcium (Ca) intakes may affect cardiovascular disease risk by altering body weight/fat and serum lipid profile, but results have been inconsistent and the underlying mechanisms are not well understood. Thus, the effects of dietary Ca on body composition and lipid metabolism were examined in male Sprague-Dawley rats. Rats were fed high-fat, high-energy diets containing (g/kg) low (0.75Ca, 0.86 ± 0.05; 2Ca, 2.26 ± 0.02), normal (5Ca, 5.55 ± 0.08) or high (10Ca, 11.03 ± 0.17; 20Ca, 21.79 ± 0.15) Ca for 10 weeks. At the end of the study the 0.75Ca group had lower ($p < 0.05$) body weight and fat mass compared to other groups. Rats fed the high Ca diets had lower serum total and LDL cholesterol compared to rats fed normal or low Ca. Liver total cholesterol was lower in rats fed high compared to low Ca. In general, liver mRNA expression of the *LDLR* and genes involved in cholesterol synthesis (*HMGCR* and *HMGCS1*), fatty acid oxidation (*CPT2*) and cholesterol esterification (*ACAT2*) were higher in rats fed higher Ca. Apparent digestibility of total trans, saturated, monounsaturated and polyunsaturated fatty acids was lower in rats fed the high compared to the low Ca diets, but the differences were greatest for trans and saturated fatty acids. Fecal excretion of cholesterol and total bile acids was highest in rats fed the 20Ca diet. The results suggest little effect of dietary Ca on body composition unless Ca intakes are very low. Decreased bile acid reabsorption and reduced absorption of neutral sterols and trans and saturated fatty acids may contribute to the improved serum lipid profile in rats fed higher Ca.

Dedication

This thesis is dedicated to my parents, sisters and brothers who supported me throughout my studies.

"There is only one thing that makes a dream impossible to achieve، the fear of failure"

Paulo Coelho

Acknowledgments and Contributions

I wish to thank my beloved family most sincerely for their unconditional love and support while I have pursued my academic studies away from home for the last number of years. Special thanks to my mother and father for their belief in me. I would like to express my thanks to Dr. Mohammad Alaraby for his continuous love and support. I would like to express my sincere gratitude to all of those who supported and guided my research and helped with proofreading of my MSc thesis. A special thanks to my sisters Dr. Wijdan Alomaim and Ms. Hayam Alomaim. You are the best sisters any one could ask for and just the feeling that you are both standing by my side gives me courage to overcome any challenges. A special thanks to my best friends Saeedah, Rajaa and Randa for their continued support during this outstanding learning journey. I am grateful to Dr. Ashok Kumar (supervisor) and Dr. Jesse Bertinato (co-supervisor) for all their help and guidance. I would like to thank Mr. Philip Griffin, Ms. Eleonora Swist, Ms. Michelle Vandeloo, and Dr. Isabelle Demonty for assistance with experiments and guidance throughout my thesis. I would like to thank my Thesis Advisory Committee, Dr. Amanda MacFarlane and Dr. Kevin Cockell for their guidance. I also would like to thank the technicians in the Scientific Services Division, Health Canada for care of the rats and measurement of food consumption, body weight and body composition.

Contributions

Dr. Jesse Bertinato conceived the study.

Food consumption, body weight and body composition measurements (by MRI) were performed by technicians in the Scientific Services Division, Health Canada.

Dominique Patry (technician, Scientific Services Division, Health Canada) measured the serum lipids and urine creatinine.

Philip Griffin and Louise Plouffe (technicians, Nutrition Research Division, Health Canada) provided technical expertise for the mineral analyses using ICP-OES.

Philip Griffin and Eleonora Swist (technicians, Nutrition Research Division, Health Canada) developed the method for measurement of neutral sterols and bile acids in feces and provided assistance with the fatty acid, neutral sterol and bile acid analyses using gas chromatography. They also provided guidance with the gene expression experiments.

Michelle Vandeloo (Co-op student, University of Ottawa) assisted with the measurement of lipids in liver.

Dr. Jesse Bertinato and Eleonora Swist provided guidance with the statistical analyses.

List of Abbreviations

ACAT	Acyl CoA-cholesterol acyltransferase
AI	Adequate intake
Apo	Apolipoprotein
ApoA1	Apolipoprotein A-1
ApoB100	Apolipoprotein B100
BA	Bile acid
BCAA	Branched-chain amino acid
BMI	Body mass index
Ca	Calcium
CA	Cholic acid
CaSR	Calcium sensing receptor
CD36	Cluster of differentiation 36
CDCA	Chenodeoxycholic acid
CE	Cholesterol ester
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
CPT2	Carnitine palmitoyl transferase-2
CR	Cholesterol retained
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7α-hydroxylase
DCA	Deoxycholic acid
DRVs	Dietary reference values
EAR	Estimated average requirement
ER	Endoplasmic reticulum
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FA	Fatty acid
FC	Free cholesterol
FFA	Free fatty acids
GI	Gastrointestinal tract
HDL	High density lipoprotein
HDL-C	HDL-cholesterol
HMGCoA	3-hydroxy-3 methylglutaryl CoA
HMGCoAR	3-hydroxy-3-methylglutaryl CoA reductase
HMGCoAS	3-hydroxy-3-methylglutaryl CoA synthase
IDL	Intermediate density lipoprotein
IOM	Institute of Medicine
K	Potassium
LCA	Lithocholic acid
LDL	Low density lipoprotein
LDL-C	LDL-cholesterol
LDLR	Low density lipoprotein receptor
Mg	Magnesium
MUFA	Monounsaturated fatty acid

γ-MCA	γ-Muricholic acid
α-MCA	α-Muricholic acid
β-MCA	β-Muricholic acid
ω-MCA	ω-Muricholic acid
NAFLD	Non-alcoholic fatty liver disease
NPC1L1	Niemann-Pick C1-like
NS	Neutral sterol
P	Phosphorus
PL	Phospholipid
PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acids
RCT	Randomized controlled trials
RDA	Recommended dietary allowance
SFA	Saturated fatty acid
TC	Total cholesterol
TFA	Trans fatty acid
TG	Triacylglycerol
UL	Tolerable upper intake level
VLDL	Very low density lipoprotein
WHO	World Health Organization

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1. Introduction

1.1 Calcium (Ca)

1.1.1 Ca is an essential mineral nutrient

Ca is an essential mineral nutrient. Over 99% of Ca in the human body is found in bone and teeth. The other 1% is present in extracellular fluid and other tissues. Ca has an important role in numerous physiological processes (Table 1). It has a vital role in formation of bone and teeth, nerve transmission (Braet et al. 2004), smooth muscle contraction (Perrino 2016), blood clotting, blood pressure regulation (Bristow et al. 2015), sperm motility (Correia et al. 2015) and insulin secretion (Santulli et al. 2015). Ca functions as a second messenger bound to calmodulin (Spitzer 2008) and is important for signal transduction (Clapham 2007).

Many neurological conditions are associated with hypocalcemia (low serum Ca concentration) such as seizures, delirium and tetany (Han et al. 2015). Hypercalcemia (high serum Ca concentration) can cause kidney stones, malignancy and hyperparathyroidism (Moe 2008).

1.1.2 Whole-body Ca homeostasis

The intestine is a major site controlling whole-body Ca balance (Figure 1). Ca is absorbed in the intestine by two pathways, an active transcellular pathway and a paracellular passive process (Bronner 2003). Transcellular absorption occurs mostly in the duodenum and upper jejunum. In passive diffusion Ca is absorbed between mucosal cells. Paracellular absorption occurs throughout the intestinal tract (Bronner 2003).

Table 1. Ca-dependent physiological processes.

Formation of bone and teeth
Blood clotting
Muscle contraction
Nerve transmission
Blood pressure regulation
Sperm motility
Hormone secretion
Cell differentiation
Immune function
Apoptosis

Ca is an essential mineral nutrient that plays an important role as a second messenger and signal transducer. Listed are some physiological processes that require calcium.

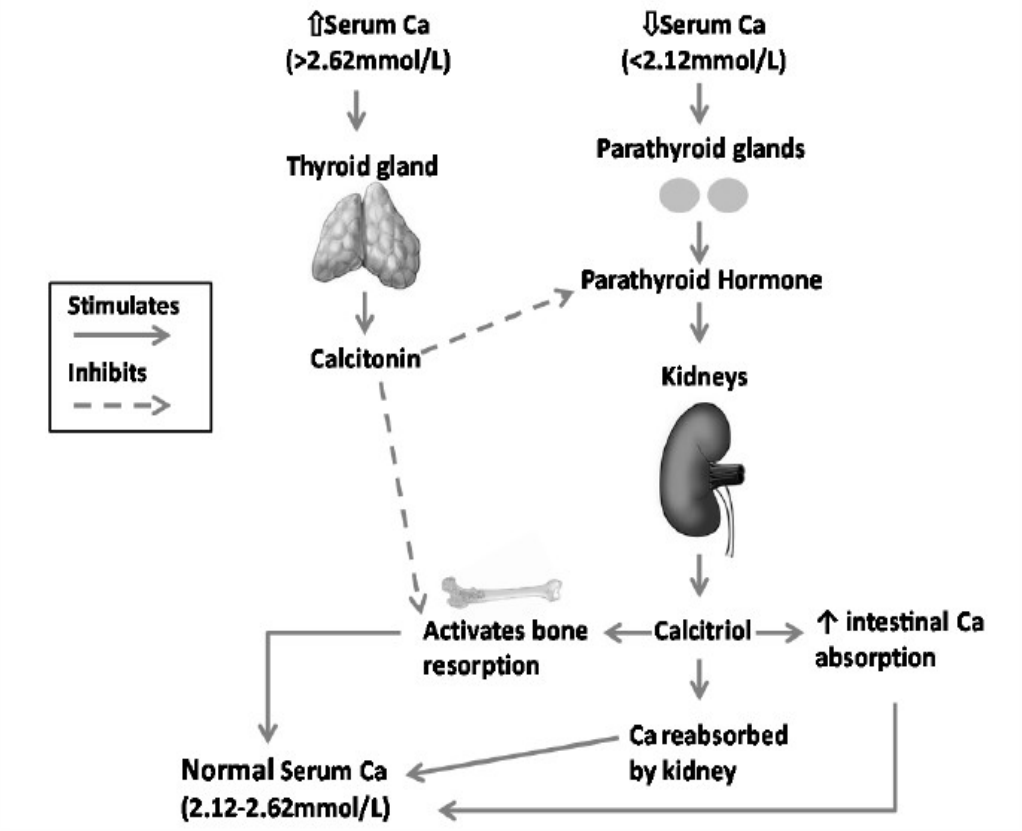


Figure 1. Whole-body Ca homeostasis. The bone, kidney and intestine are the major organs regulating serum ionized Ca concentrations. Under conditions of Ca deficiency a decrease in serum Ca stimulates PTH release from the parathyroid gland. PTH acts at the kidneys to produce the active form of vitamin D (calcitriol). Calcitriol triggers intestinal absorption of Ca and release of Ca from bone increasing serum Ca concentrations. Under conditions of excess Ca elevated serum Ca stimulates calcitonin release from the thyroid gland. The calcitonin counteracts the action of PTH and inhibits Ca resorption from bone decreasing serum Ca concentrations. Figure adapted from: (Smolin, Grosvenor, & Garfinkel 2015).

When dietary Ca intake is low the transcellular pathway accounts for a significant fraction (80%) of the absorbed Ca (Pu et al. 2016). When consumption of Ca is high, the active transcellular pathway accounts for only a small fraction of the absorbed Ca. This is due to the down-regulation of Ca transport proteins in intestinal absorptive cells with high dietary Ca (Pu et al. 2016).

The kidneys and bone also play major roles in controlling whole-body Ca balance. Serum Ca concentration is under tight homeostatic regulation and must be maintained within a narrow range. The normal range for humans is 8.5 and 10.5 mg/dL. Bone acts as a Ca store that supplements the serum under conditions of Ca deficiency (Ross et al. 2011). When Ca intakes are high the kidneys function to remove excess body Ca in the urine (Ross et al. 2011). The kidneys control Ca loss in the urine by regulating the reabsorption of Ca in the renal tubules. Ca loss from the body is mainly in the urine and feces. Other routes such as sweat contribute minimally to Ca loss.

Parathyroid hormone (PTH), vitamin D, and calcitonin are important hormones regulating serum Ca concentrations. Calcium sensing receptors (CaSR) in the parathyroid gland sense when there is a decrease in serum Ca concentration. This leads to an increase in the secretion of PTH by the parathyroid gland. Higher PTH stimulates 1α-hydroxylase activity in the kidney which converts 25-hydroxyvitamin D to the active form 1,25 dihydroxyvitamin D (also called calcitriol). This increases intestinal Ca absorption and stimulates release of Ca from bone. There is also inhibition of renal Ca excretion. The overall result is a rise in serum Ca concentration. When Ca intake is high and serum Ca is elevated it results in a decrease in PTH production by the parathyroid gland. This results in elevated urinary Ca excretion. Under conditions of increased serum Ca concentrations, the thyroid gland secrets calcitonin, a hormone

that counteracts the effects of PTH and inhibits resorption of Ca from bone decreasing serum Ca concentrations (Ross et al. 2011).

1.1.3 Interaction of Ca with phosphorus (P) and magnesium (Mg)

Metabolism of Mg and P are dependent on Ca. Mg is considered the second most plentiful intracellular cation after potassium (K) and functions in many diverse physiological processes (Bertinato 2017). Mg is involved in nerve transmission, muscle contraction/relaxation and insulin and glucose metabolism (Bertinato 2017). Low Mg intakes and serum concentrations have been associated with a number of chronic diseases and health conditions including migraine headaches, inflammation, stroke, Alzheimer's disease (Volpe 2013), cardiovascular disease, type 2 diabetes mellitus, hypertension and osteoporosis (Costello et al. 2016). Studies have shown that high Ca intakes increase the excretion of Mg in urine and decrease intestinal Mg absorption (Bertinato et al. 2016). This is likely explained by competition between Ca and Mg for intestinal absorption and renal reabsorption.

As discussed above a decrease in serum Ca concentration stimulates the secretion of PTH. PTH restores Ca concentrations in the serum by stimulating osteoclastic breakdown of bone which releases Ca and also P into the circulation. Higher PTH also acts at the kidneys to inhibit renal excretion of Ca but also increase renal excretion of P. The increased production of calcitriol by PTH stimulates intestinal absorption of both Ca and P (Digirolamo et al. 2012).

1.1.4 Dietary reference values for Ca

Dietary reference values (DRVs) for Ca for the North American (Canada and US) population were updated in 2010 by the Institute of Medicine (IOM) (Ross et al. 2011). Estimated average requirements (EAR), recommended dietary allowances (RDA), adequate intakes (AI) and tolerable upper intake levels (UL) have been established (Table 2). The EAR is defined as the estimated level of nutrient intake that meets the requirement of half the population. The RDA is defined as the estimated level of nutrient intake that meets the requirement of almost all of the population (i.e., 97.5%). The UL is the highest level of nutrient intake that is expected to have no adverse effects for almost all the population. The AI is a recommended daily intake based on estimates of nutrient intakes that are presumed adequate. An AI is set when there is not enough data to set an EAR (and RDA) for a nutrient.

Dietary requirements for Ca were based on the need for bone accretion, bone mass stability and prevention of bone loss. For most sex-age groups an EAR and RDA has been established. A UL has been established for all sex-age groups and is based on long-term intervention studies on the adverse health effect of consuming high Ca on the development of kidneys stones. Reference values for specific life-stage groups are shown in Table 2. For adults the EAR ranges from 800–1000 mg per day and the UL ranges from 2000–2500 mg per day. For infants an AI has been established based on the amount of Ca in breast milk and the amount of breast milk that is assumed to be sufficient for an infant. It is important to note that the UL is only modestly higher than the RDA for Ca (less than 2-fold for older adults).

Table 2. Dietary reference values for Ca.

Life-stage group	EAR mg/day	RDA mg/day	UL mg/day
Infants (0-6) mo			1000
Infants (6-12) mo			1500
1-3 y	500	700	2500
4-8 y	800	1000	2500
9-13 y	1100	1300	3000
14-18 y	1100	1300	3000
19-30 y	800	1000	2500
31-50 y	800	1000	2500
51-70 y males	800	1000	2000
51-70 y females	1000	1200	2000
>70 y	1000	1200	2000

EAR, estimated average requirement; mo, months; RDA, recommended dietary allowance; UL, tolerable upper intake level; y=years. Data obtained from: (Ross et al. 2011).

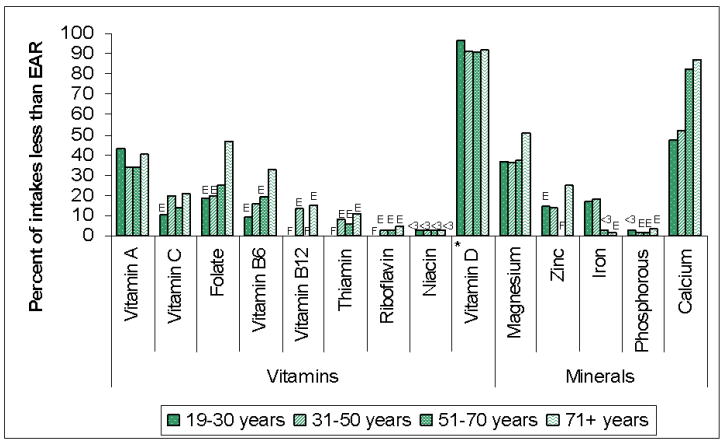
1.1.5 Ca intakes

Food sources that are rich in Ca include dairy product such as milk, cheese and yogurt. Some types of water (mineral and spring water) can also provide significant amounts of Ca (Bertinato & Taylor 2013). Consumption of one serving of foods with the highest Ca content can provide up to 50% of the RDA for adults aged 19–50 years. Ca supplements can also be a significant source of Ca for supplement consumers (Martineau et al. 2014). Common Ca supplements contain between 65–1500 mg of Ca (Health Canada, 2016). This corresponds to 6.5–150% of the RDA for adults aged 19–50 years. The use of high-dose Ca supplements is common among older women for maintaining bone health (Martineau et al. 2014).

In Canada, results from the Canadian Community Health Survey 2.2 conducted in 2004 showed that a large proportion of adults 19 years and older for both sex groups have Ca intakes from food below the EAR suggesting inadequate Ca intakes for many Canadians (Figure 2A, B) (Health Canada 2012). For some sex-age groups the percent of intakes of Ca less than the EAR exceeds 50%. The results also suggest that Ca intakes compared to dietary recommendations are lower for older adults.

The reported low Ca intakes relative to requirements raise suspicions of widespread Ca deficiency in Canada that may increase the risk for diseases including osteoporosis (Vatanparast et al. 2009). A likely explanation for the low Ca intakes in the Canadian population is changes in eating behavior (Birch et al. 2007). Canadians are replacing foods rich in Ca such as milk and other dairy products with foods low in Ca (e.g., coffee, soft drinks, sport drinks, demineralized water). Persons with lactose intolerance are more vulnerable to low Ca intakes since many foods containing lactose are also good sources of Ca such as dairy products (Heaney 2013).

A



B

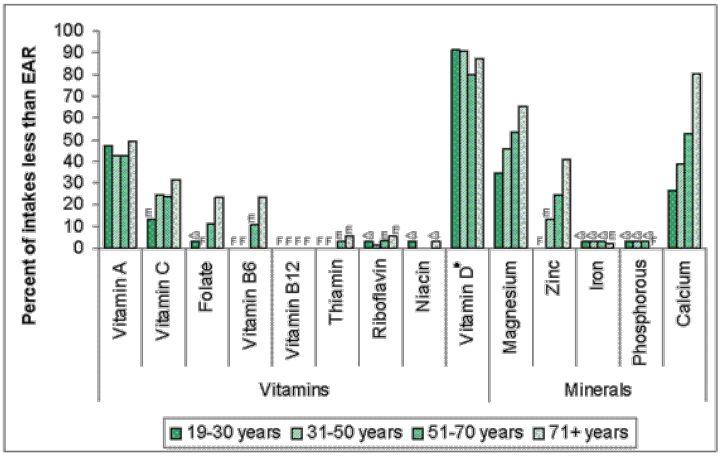


Figure 2. Percentage of Canadian adults with Ca intakes below the EAR. Percentage of Canadian women (A) and men (B) with nutrient intakes from food below the EAR. For some sex-age groups the percent of intakes less than the EAR exceed 50% for Ca. EAR, estimated average requirement. Figure adapted from: (Canadian Community Health Survey 2.2, 2004).

Although many Canadians have inadequate Ca intakes compared to current dietary recommendation some subpopulations have Ca intakes exceeding the UL from use of high-dose Ca supplements (Martineau et al. 2014). The 95th percentile of Ca intakes (from food and supplements) exceeds the UL for women 51–70 years (Martineau et al. 2014). Thus, nutrition surveys in Canada suggest that a substantial proportion of the population have inadequate Ca intakes, but some subpopulations (e.g., older women) are exceeding the UL.

1.2 Ca and obesity

1.2.1 Obesity is a global epidemic

Obesity is characterized by a positive energy balance that results from surplus energy intake and/or insufficient energy expenditure. Obesity is manifested by an excess of adipose tissue (Jung & Choi 2014). It is thought that it is not the amount of fat present but rather the functionality of the fat that determines the development of metabolic consequences of obesity. Obesity is a major public health problem worldwide (Zagotta et al. 2015). For adults, obesity, overweight and normal weight is defined as a body mass index (BMI) of 18.5–<25, 25–<30 and ≥30.0 kg m^2, respectively (Villarroel et al. 2014). For rats established cut-off values for obesity, overweight and normal weight have not been established.

Obesity increases the risk of developing many diseases and health conditions including cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes, hypertension, dyslipidemia and some types of cancers (Villarroel et al. 2014). It is estimated that obesity in the near future will exceed smoking as the leading cause of preventable death (Schrager 2005).

1.2.2 Ca intake and body composition

Many factors can influence the risk for obesity including genetic factors and non-genetic factors including age, medical problems and diet (Griffin & Lichtenstein 2014). Ca intake may also modify the risk for obesity through its extra skeletal effects. Several mechanisms have been proposed to explain the observed inverse relationship between Ca intake and body weight and/or body fat reported in some studies (Figure 3) (Villarroel, P. et al., 2014). It has been proposed that the lower circulating PTH and 1,25 dihydroxyvitamin D concentrations with higher intakes of Ca causes a decrease in cytosolic Ca in adipocytes. This decrease in cytosolic Ca triggers a downregulation of lipid storage by decreasing lipogenesis and increasing lipolysis and fat oxidation (Soares et al. 2014). Another proposed mechanism by which Ca may regulate body weight and body fat is through interaction with dietary fats in the gastrointestinal tract (GI). Ca can bind fatty acids (FA) in the GI tract forming insoluble Ca-fatty acid soaps that are excreted in the feces (Soares et al. 2014). Since FA complexed with Ca are unavailable for absorption the digestible energy from the diet would be expected to be lower with higher Ca intakes. It has also been proposed that higher Ca intakes may reduce appetite (Soares et al. 2014).

Studies in humans have reported an inverse relationship between Ca intake and body weight/fat (Davies et al. 2000; Heaney et al. 2002; Zemel et al. 2004; Zemel et al. 2005), but not all studies have reported this effect (Gunther et al. 2005; Reid et al. 2005; Shapses et al. 2004; Bowen et al. 2005). In a human study by Periera et al consumption between 800–1500 mg of Ca per day in adults aged 18–30 years showed an inverse relationship between dairy products and obesity (Pereira et al. 2002). In a randomized controlled study by Shalileh et al 40 adults consumed a calorie restricted diet and either 1000 mg per day of Ca (as calcium

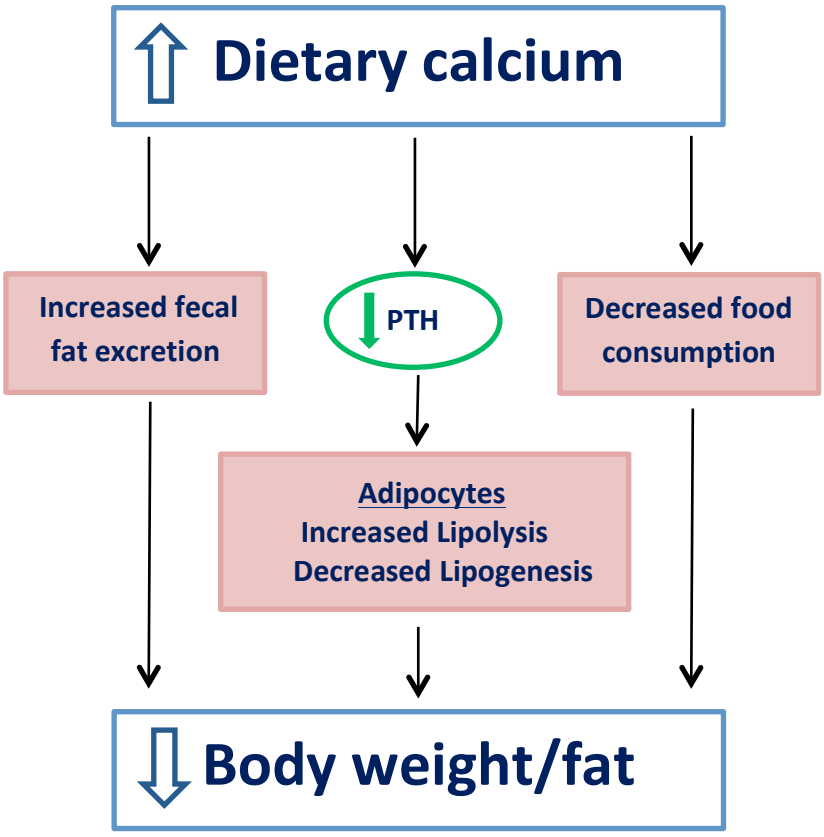


Figure 3. Illustration of the proposed mechanisms to explain the beneficial effect of higher Ca intakes on body weight and fat mass. An increase in dietary Ca causes a reduction in PTH in the circulation. This causes a decrease in cytosolic Ca in adipocytes through a decrease in 1,25 dihydroxyvitamin D. The decrease in cytosolic Ca causes a downregulation of genes involve in lipid storage and an increase in expression of genes involved in lipolysis and fat oxidation. Higher Ca intakes may also decrease appetite and promote fecal fat excretion and energy loss from the diet.

carbonate) or placebo for 24 weeks (Shalileh et al. 2010). The study showed no effect of Ca supplementation on body weight or body composition.

There are several factors that may explain the inconsistent results reported in studies. First, the effect of Ca intake on body weight and body fat may be small. Second, a larger effect may only occur when baseline Ca intakes are low (suggesting a Ca deficiency). It has also been suggested that increased Ca intake may have a larger effect when coupled with reduced calorie intake. Lastly, studies suggest that Ca from dairy products have a stronger effect on reducing body weight or fat than Ca alone (Thomas et al. 2012). This may be explained by additional anti-obesity compounds in dairy such as branched-chain amino acids (BCAA) (Zemel 2004).

Studies on the effects of dietary Ca on body weight and fat mass in rats and mice have also produced mixed results (Papakonstantinou et al. 2003; Han He et al. 2011; Marotte et al. 2014; Bollen & Bai 2005). In a study by Papakonstantinou et al 24 male Wistar rats were fed a low Ca diet (0.2%) or high Ca diet (2.2%) from non-fat dry milk for 85 days (Papakonstantinou et al. 2003). The results showed that rats fed the higher Ca diet had reduced body weight and body fat. In another study, 90 Sprague-Dawley rats were fed diets containing normal (0.5%), low (0.30%) or very low (0.15%) Ca for 15 weeks (Han He et al. 2011). The results showed that rats fed the very low Ca diet had greater adipose tissue compared to rats fed the normal Ca diet. Marotte et al fed male mice a high Ca diet (1.2%), a high vitamin D diet or a high Ca (1.2%), high vitamin D diet for 10 weeks (Marotte et al. 2014). The results showed that the three diets reduced body weight and body fat and the effect was greater for the group fed the high Ca, high vitamin D diet. Bollen et al studied the effect of long-term high Ca intake on body weight and body fat in male and female Sprague-Dawley rats (Bollen & Bai 2005). Rats were fed diets containing high Ca (1%) or low Ca (0.25%) for 34 weeks. The results showed that male rats fed

high Ca had reduced body weight and body fat. Female rats also had reduced body weight and body fat up to week 13 of the study. Malekzadeh et al fed male Sprague-Dawley rats a low (0.2%), normal (0.5%) or high (1.2%) Ca diet for 10 weeks (Malekzadeh et al. 2007). The results showed no significant differences among groups in body weight, body fat or food intake. Zhang et al fed female mice and Sprague-Dawley rats a low (0.2%), normal (0.6%) or high (1.8%) Ca diet for 10−12 weeks. The results did not support an effect of dietary Ca on body weight, body fat or energy intake. Thomas et al fed mice high-fat diets containing normal Ca (0.5%), high Ca (1.5%) or high Ca (1.5%) plus non-fat dry milk (Thomas et al. 2012). Compared to mice fed normal Ca, the high Ca diet increased body weight, body fat and energy intake of the mice. However, the high Ca plus non-fat dry milk diet reduced body weight and body fat compared to mice fed normal Ca. The results suggest that other components in the non-fat dry milk (i.e., not the Ca) had anti-obesity properties.

Since obesity is affected by many factors, any one factor likely only makes a small contribution. Even though the effect of Ca intake on obesity may be small for any one individual, even a small effect can have meaningful implications on health at the population level. Thus, given the inconsistent results in human and animal studies regarding the effects of Ca intakes on body weight and body composition, further research is warranted to address this issue.

1.3 Lipid metabolism

1.3.1 Intestinal lipid absorption

Triacylglycerols (TG), cholesterol esters (CE) and phospholipids (PL) are the major lipids found in food. TG are compounds made up of a glycerol backbone and three FA. A critical phase in the digestion of TG is emulsification with bile salts. Emulsification significantly

increases the fat surface area that facilitates hydrolysis by lipases in the intestinal lumen. Hydrolysis releases free fatty acids (FFA) and monoacylgycerols that are absorbed by the enterocytes by diffusion or protein-mediated transport mechanisms involving fatty acid transport proteins and cluster of differentiation 36 (CD36) (Ros 2000; Ravid et al. 2008). Once absorbed into enterocytes, FFA can be transported to the endoplasmic reticulum (ER). In the ER monoacylglycerols are esterified with FFA to form TG. TG can be incorporated into chylomicrons for secretion into the circulation.

CE from the diet are hydrolyzed to FFA and free cholesterol (FC). Niemann-Pick C1-like (NPC1L1) plays a major role in the absorption of cholesterol in enterocytes (Ros 2000; Ravid et al. 2008). FC once absorbed into enterocytes can be excreted back into the intestinal lumen by the action of ABCG5/ABCG8 (Barona & Fernandez 2012; Ravid et al. 2008). FC in enterocytes can be esterified in the ER into CE by acyl CoA-cholesterol acyltransferases (ACAT). Cholesterol can then be secreted into the circulation in chylomicrons or high density lipoproteins (HDL). Chylomicrons contain FC and CE. HDL contains mostly FC.

Insulin is a hormone produced by beta cells of the pancreatic islets. Insulin promotes the absorption of glucose into cells and regulates the metabolism of carbohydrates, fats and protein. In insulin resistance, organs (e.g., liver, adipose tissue and muscle) do not respond normally to insulin. Thus, more insulin is needed to help cells uptake glucose.

Insulin resistance is associated with dyslipidemia and higher risk of cardiovascular disease (CVD) (Patel et al. 2016). Dyslipidemia in insulin resistance is characterized by higher serum concentrations of triglyceride-rich lipoproteins and lower serum HDL cholesterol concentrations. Studies suggest that intestinal insulin resistance increases chylomicron production and may decrease HDL production (Hussain 2015).

1.3.2 Lipoprotein metabolism

Lipoproteins are lipids combined with protein. Lipoproteins contain CE and TG located in the central core enveloped by FC, PL, and apolipoproteins (Apo). The protein moieties of lipoproteins are recognized by receptors on the surface of cells. Lipoproteins are mainly produced by the small intestine and the liver (Figure 4). The small intestine releases chylomicrons into the circulation. After utilization of lipids by peripheral tissues, chylomicron remnant particles are taken up by hepatocytes in the liver. The liver releases very low density lipoprotein (VLDL) and premature HDL into the circulation. In the circulation, VLDL is hydrolyzed and converted into intermediate density lipoproteins (IDL) and low density lipoprotein (LDL). LDL and IDL can be taken up by hepatocytes. HDL plays an important role in reverse cholesterol transport that moves cholesterol from peripheral tissues to the liver via a multi-step process (Jiang et al. 2013).

When the diet contains more FA than are needed immediately for fuel or as precursors, the liver converts them to TG. The TG are secreted by the liver in VLDL and transported in the circulation to adipose tissue where they are stored in lipid droplets within adipocytes. VLDL is the only route for secretion of TG from hepatocytes.

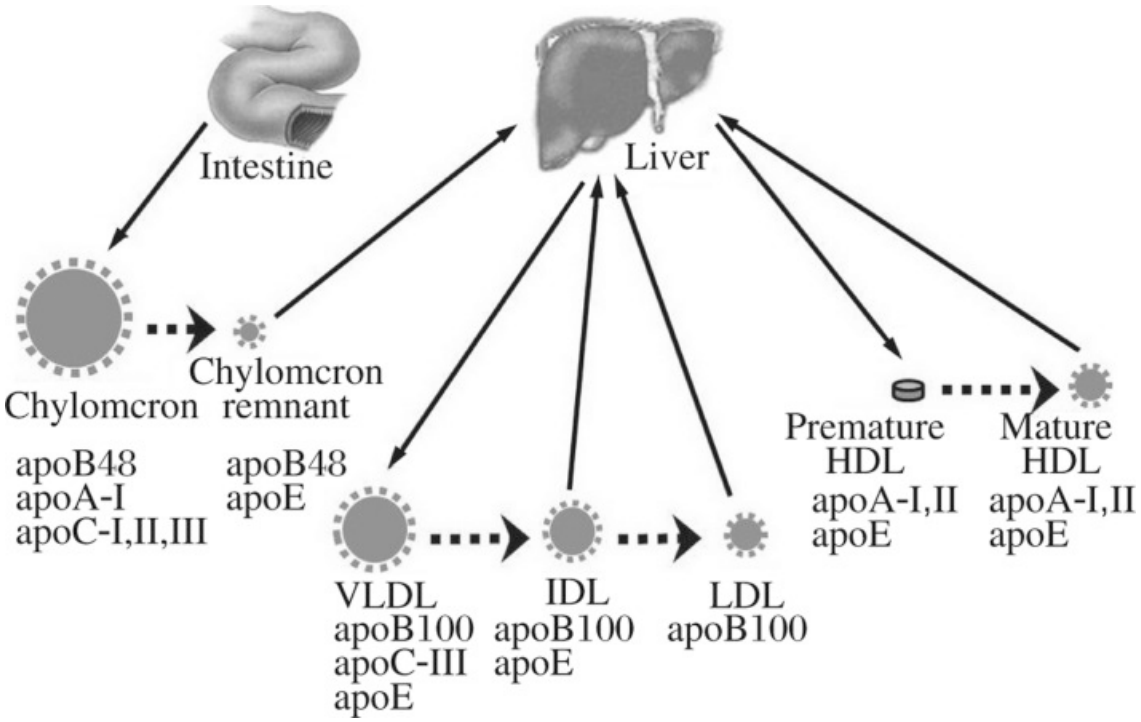


Figure 4. Lipoprotein metabolism. The main sites for lipoprotein production are the small intestine and the liver. Chylomicron are produced by the intestines. Once the lipids are distributed to peripheral tissues the remnant particles are taken up by the liver. VLDL and premature HDL are secreted from the liver. VLDL is converted to IDL and LDL in the circulation. The liver takes up the IDL and LDL from the circulation. Premature HDL is converted to mature HDL in the circulation which plays a critical role in reverse cholesterol transport through the delivery of cholesterol from the peripheral tissues back to the liver. Figure adapted from: (Jiang et al. 2013).

1.3.3 Lipid metabolism in the liver

The liver is responsible for cholesterol homeostasis (Lecerf & Lorgeril 2011). The total liver cholesterol pool is affected by cholesterol uptake, de novo synthesis, and the conversion of cholesterol to bile acids (BA) for secretion (Stellaard & Lütjohann 2017) (Figure 5). Cholesterol is present in all tissues and can be produced by the liver but can also be obtained from the diet (animal food sources). Cholesterol serves many vital functions in the body including for steroid hormone production, generation of vitamin D and synthesis of BA.

Low density lipoprotein receptors (LDLR) are found on the surface of liver cells and recognize apolipoprotein B100 (ApoB100), which is a component of LDL particles. After binding ApoB100, the LDLR and LDL particles move into the cell by endocytosis. Following the uptake of LDL cholesterol from the circulation some of the absorbed cholesterol is converted into CE by ACAT which attaches a FA to the free hydroxyl group in the cholesterol molecule.

Acetyl CoA is a precursor for cholesterol synthesis. The first step in cholesterol synthesis is the formation of 3-hydroxy-3 methylglutaryl CoA (HMGCoA) from acetyl CoA by the action of 3-hydroxy-3-methylglutaryl CoA synthase (HMGCoAS), the rate-limiting enzyme in cholesterol synthesis. This product is reduced to mevalonate by the action of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCoAR) which is then converted to cholesterol (Griffin & Lichtenstein 2014).

Acetyl CoA is also a precursor for FA synthesis which occurs in the cytoplasm. Fatty acid synthase (FAS) is a multi-complex enzyme that functions in the biosynthesis of saturated fatty acids (SFA). β-oxidation of FA to generate energy occurs in the mitochondria. Carnitine

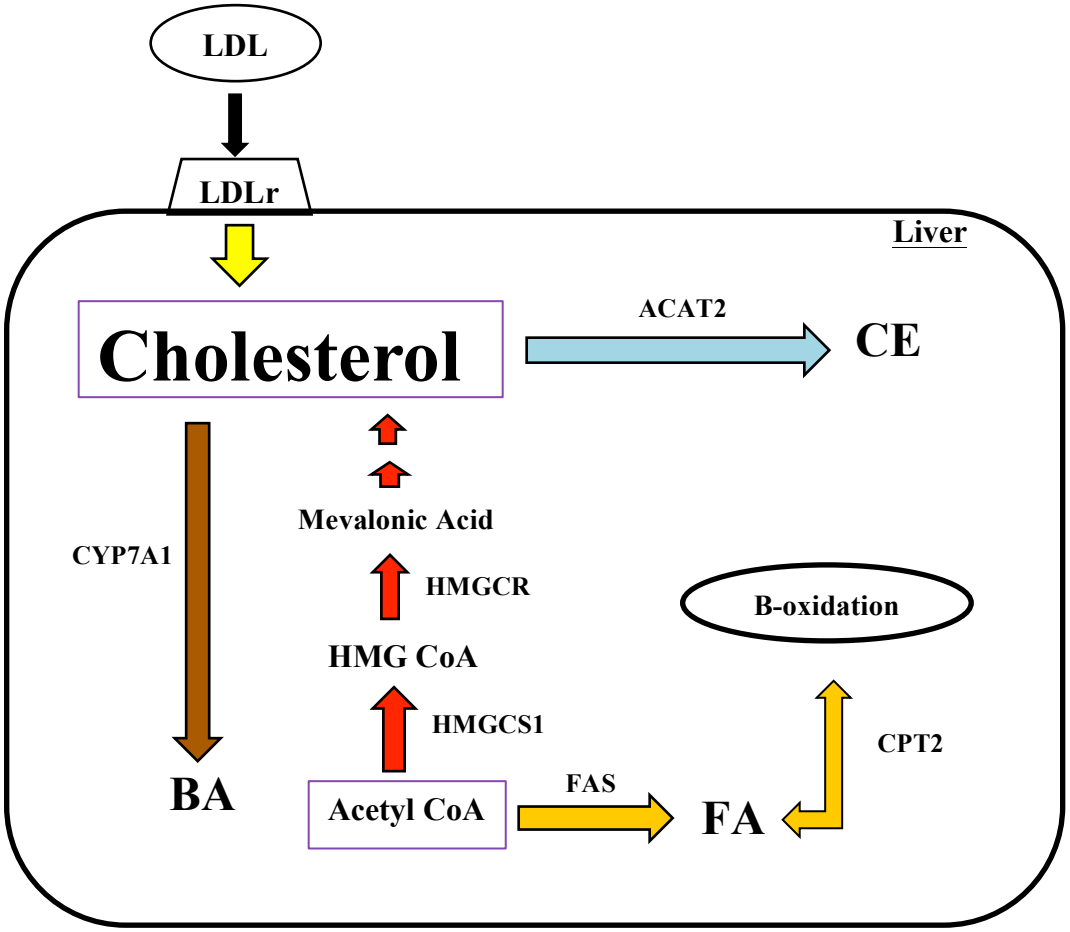


Figure 5. Lipid metabolism in the liver. Liver cells express the LDLr for the uptake of LDL from the circulation. Following the release of free cholesterol into the cytoplasm, some is esterified by ACAT2 to form CE. Some of the cholesterol is converted to natural BA by CYP7A1. Acetyl CoA is a precursor for cholesterol and FA synthesis. The rate-limiting enzyme in cholesterol synthesis is HMGCS1 that catalyzes the formation of HMG CoA. HMG CoA is converted to mevalonate by the action of HMGCR. The rate-limiting multi-complex enzyme in FA synthesis is FAS. CPT2 is involved in FA β-oxidation.

palmitoyl transferase-2 (CPT2) catalyzes the formation of acyl CoA to start the β-oxidation cycle.

BA have many important functions such as aiding in the digestion of fat. Primary BA are synthesised in the liver from cholesterol. BA synthesis starts with the conversion of cholesterol to 7α hydroxycholesterol by the action of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the classic BA synthesis pathway. Primary BA secreted into the intestine can be converted into secondary BA from the action of bacteria. A large proportion (~95%) of BA secreted into the intestine are re-absorbed back to the liver through enterohepatic circulation. Under normal conditions only a small fraction of the BA are ultimately excreted in the feces (Figure 6). Conditions that reduce the re-absorption of BA (i.e., increase fecal excretion) can have profound effects on cholesterol metabolism since more cholesterol would be required to replace loss BA.

1.4 Lipids and CVD

1.4.1 Blood lipids: risk factors for CVD

CVD is class of disorders that affect the heart or blood vessels such as coronary heart disease (CHD). Blood lipids are well-established risk factors for CVD. Higher concentrations of serum total cholesterol (TC) and LDL-cholesterol (LDL-C) and TG increase the risk for CVD (Sampson et al. 2013), while lower concentrations of HDL-cholesterol (HDL-C) increase CVD risk (Sampson et al. 2013). Higher serum TC:HDL-C and LDL-C:HDL-C ratios are also positively correlated with risk of CVD (Waldmann et al. 2017). Lowering specifically LDL-C has been shown to significantly reduce the risk of CHD. HMGCoA reductase inhibitors

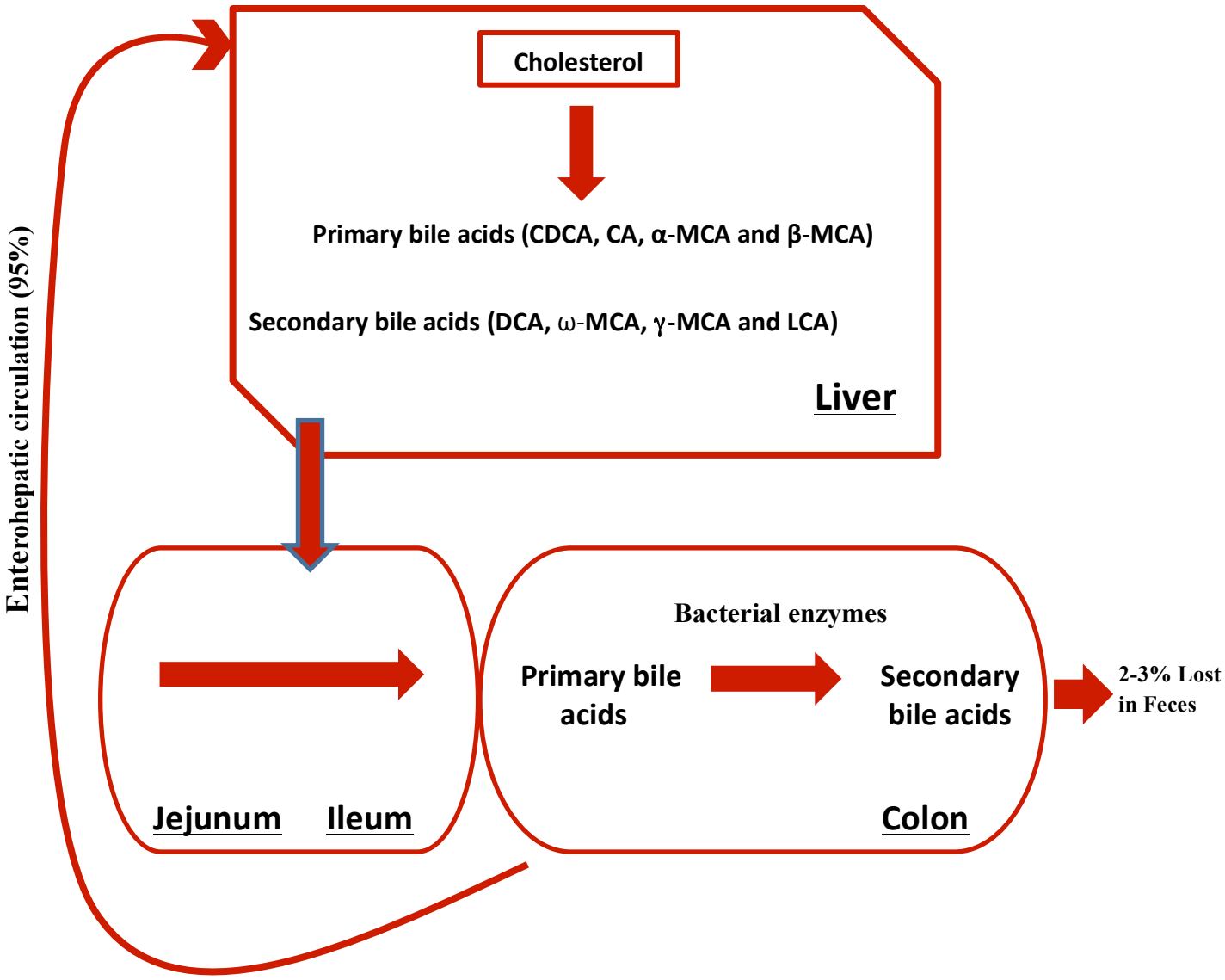


Figure 6. Enterohepatic circulation of BA. The process involves synthesis of primary BA from cholesterol. Primary BA are conjugated to either taurine or glycine and released into the intestinal lumen to help with digestion. Intestinal bacteria form secondary BA from primary BA. Primary and secondary BA in the intestinal lumen are efficiently (~95%) re-absorbed back to the liver. Under normal conditions only a small amount of BA are excreted in the feces (2–3%). CDCA, chenodeoxycholic acid; CA, cholic acid; α-MCA, α-muricholic acid; β-MCA, β-muricholic acid; γ-MCA, γ-muricholic acid; ω-MCA, ω-muricholic acid; LCA, lithocholic acid; DCA, deoxycholic acid.

('statins') are used to treat hypercholesterolemia and decrease the risk for CHD (Sampson et al. 2013).

1.4.2 Fatty acids (FA) and CVD

FA can be classified into four major types including saturated fatty acids (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acids (PUFA) and trans fatty acids (TFA) (Figure 7). FA can be distinguished based on different properties such as the number of double bonds. SFA contain no double bonds. Unsaturated fatty acids can be divided into two classes MUFA that have only one double bond and PUFA that have at least two double bonds.

The structure of the double bonds in unsaturated fatty acids can be 'cis' when the hydrogen atoms line in the same direction or 'trans' when the hydrogen atoms line in the opposite direction. The 'cis' configuration allows the FA to bend more easily compared to the 'trans' configuration. FA also differ in the location of the double bonds and the length of the carbon chain (Mensink 2016).

Different types of FA have different effects on blood lipids and CVD risk (Brouwer 2016). TFA have the most detrimental effect on blood lipids. TFA raise serum LDL-C and lower HDL-C levels (Krenosky et al. 2012). In the mid-1990's it was estimated that Canadians had one of the highest average TFA intakes in the world (~3.7% of energy). The World Health Organization (WHO) recommends that TFA intakes should be less than 1% of total energy intake (Brouwer 2016). Since there is a linear positive relationship between TFA intake and serum LDL-C, even very low TFA intake may increase the risk of CVD. Replacing TFA with other type of FA such as MUFA or PUFA and also SFA has a beneficial effect on blood

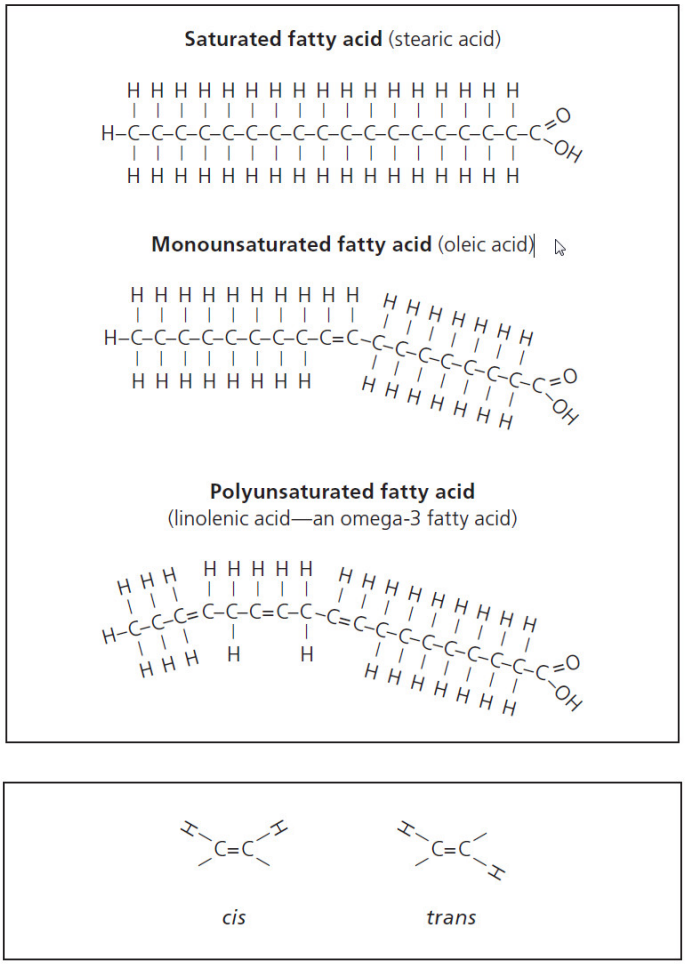


Figure 7. Structures of FA. FA can be defined based on bonds and the number of double bonds. For example, SFA have no double bonds, MUFA have one double bond, and PUFA have more than one double bond. The structure of the double bonds in unsaturated fatty acids can be cis when the hydrogen atoms line in the same direction or trans when the hydrogen atoms line in the opposite direction. Figure adapted from: (White 2009).

Lipid profile. Replacing TFA with MUFA or PUFA has been shown to decrease serum TC, LDL-C and TG (Brouwer 2016).

TFA can be divided into industrial or natural (also called ruminant) TFA. Industrial TFA are formed during hydrogenation processes or inadvertently during oil refining. Natural TFA are derived from ruminant sources and are found in larger proportions in milk and other dairy products. Both industrial and natural TFA are found in foods but the proportions in one product versus another vary. For example, the natural TFA vaccenic acid is present in higher proportions in food products that come from ruminant sources such as milk and other dairy products. The adverse effects of industrial TFA consumption on CVD risk is well documented (Stender 2015). The effects of naturally occurring TFA are less clear (Stender 2015). It is possible that natural TFA are less harmful than industrial TFA or have the same adverse effects but are simply consumed in lower quantities. Some studies also suggest possible beneficial effects of natural TFA on CHD (Stender 2015).

The second worse FA for blood lipid profile is SFA. Many studies in animals and humans have shown adverse effects of SFA on serum lipid profile. Replacing SFA with MUFA or PUFA in the diet has been shown to reduce serum TC, LDL-C and TG (Brouwer 2016). SFA have a detrimental effect on blood lipids by increasing production of apoB100-containing lipoproteins (LDL) and by impairing the activity of the LDLR (Fernandez & West 2005). Health Canada recommends consuming as little as possible of TFA and SFA while still consuming a nutritionally adequate diet (Health Canada, 2016).

1.4.3 Cholesterol and CVD

Cholesterol from dietary source has been shown to increase serum TC and LDL-C concentrations (Griffin & Lichtenstein 2014). The same observation has been made in animals including primates (Mcgill et al. 1981), swine (Pownall et al. 1980), guinea pigs (Guo et al. 1977), dogs (Mahley et al. 1974), rabbits (Roth et al. 1983), and rats (Wang et al. 2010). However, the impact of dietary cholesterol on serum lipid profile is still controversial since there are other studies that have reported that cholesterol increases both HDL and LDL particles without changing LDL-C:HDL-C ratio which is a well-establish risk factor for CVD (Barona & Fernandez 2012). Also, dietary cholesterol has been shown to increase HDL without altering LDL particles when coupled with reduced calorie intake (Barona & Fernandez 2012).

1.5 Dietary Ca and lipid metabolism

1.5.1 Ca intake and blood lipid profile

Emerging evidence suggests that dietary Ca may play an important role in improving serum lipid profile suggesting that Ca intakes may influence the risk of CVD. Some human studies have shown improved serum lipid-lipoprotein profile with higher Ca intakes (Groot et al. 1980; Denke et al. 1993; Shahkhalili et al. 2001; Ditscheid et al. 2005), but not all studies have confirmed these findings (Reid et al. 2010; Palacios et al. 2011; Bostick et al. 2000). Oral calcium carbonate administration to children with familial hypercholesterolemia was shown to induce an increase in serum apolipoprotein A-1 (ApoA1) and a decrease in LDL-C compared to the placebo group (Groot et al. 1980). Consumption of Ca-fortified foods (total Ca intake of 2200 mg/day) lowered total and LDL-C concentrations in healthy men compared to a low Ca diet (total Ca intake of 410 mg/day) (Denke et al. 1993). In a randomized, double-blind crossover

study in men, consumption of cocoa butter with a 0.9%-Ca supplement (900 mg/day) reduced plasma LDL-C by 15% compared to the control group that did not receive the Ca supplement (Shahkhalili et al. 2001). In that study plasma HDL-C was unaffected. Calcium phosphate supplementation in women and men for 4 weeks lowered serum cholesterol (Ditscheid et al. 2005). Oral Ca supplementation (1500 mg/day) in obese males for 8 weeks reduced serum TG and increased serum HDL-C from baseline measurements (Sein et al. 2017). In that study reductions in serum total and LDL-C levels were only detected in dyslipidemic centrally obese subjects.

Results from some human studies do not support a beneficial effect of higher Ca intake on serum lipids. In a randomized, controlled trial of Ca supplementation (600 or 1200 mg Ca/day) in men for 2 years reported no effects on serum HDL-C:LDL-C ratio, TG, LDL-C or HDL-C (Reid et al. 2010). In a 21-week randomized trial in Puerto Rican obese adults a high dairy (~1200 mg Ca/day from dairy products) or high Ca (~1171 mg Ca/day from diet and supplement) diet did not alter serum total and LDL-C, TG and HDL-C compared to the control group (668 mg Ca/day) (Palacios et al. 2011). In a randomized, double-blind, placebo-controlled trial with women and men, Ca supplementation of 1000 or 2000 mg/day for 4 months did not show statistically significant differences in TC or HDL-C compared to the placebo group (Bostick et al. 2000).

Higher Ca intake has been shown to decrease serum cholesterol in hamsters (Ma et al. 2011), rabbits (Hsu & Culley 2006), rats (Olatunji et al. 2008) (Vaskonen et al. 2002) and pigs (Rodas et al. 1996). A study in obese Zucker rats has shown that increases in dietary Ca intakes from 0.2 to 0.8 to 2.1% dose-dependently decreased serum TC, LDL-C and TG and increased HDL-C and HDL-C:LDL-C ratio in rats fed a 1% cholesterol diet (Vaskonen et al. 2002). In oral

contraceptive-treated female Sprague-Dawley rats higher dietary Ca (2.5% Ca diet) prevented increases in LDL-C and TC:HDL-C and LDL-C:HDL-C ratios suggesting a Ca enriched diet has a beneficial effect on blood lipid profile (Olatunji et al. 2008). In a study with ovariectomized hamsters fed 0, 2, 6 or 8 g of Ca per kg diet showed that plasma TC, TG and non-high density lipoprotein cholesterol were dose-dependently decreased with increasing dietary Ca (Ma et al. 2011). In that study, HDL-C was increase in a dose-dependent manner with higher dietary Ca.

1.5.2 Ca intake and FA, neutral sterol (NS) and bile acid (BA) absorption

Dietary Ca may affect serum lipid profile by interacting with FA, NS or BA in the GI tract. Studies suggest that Ca binds FA in the GI tract forming insoluble Ca/fatty acid soaps reducing the absorption of FA. Consumption of Ca-fortified foods (total Ca intake of 2200 mg/day) increased the fecal excretion of saturated fats from 6 to 13% in men (Denke et al. 1993). However, in that study there was no change in fecal BA excretion. In a randomized, double-blind crossover study in men, consumption of cocoa butter with a 0.9%-Ca supplement (900 mg/day) increased fecal fat 2-fold and reduced the absorption of cocoa butter by 13.0% that was mostly due to an increase in the excretion of palmitic and stearic acids (Shahkhalili et al. 2001). Ca phosphate supplementation in women and men for 4 weeks did not change fecal fat excretion or fecal excretion of total NS (i.e., cholesterol and its transformation products) (Ditscheid et al. 2005). However, cholesterol excretion increased, while the excretion of the metabolite coprostanol decreased. Excretion of BA increased with Ca phosphate supplementation. A randomized crossover study in women and men examined the effect of high Ca from dairy products (2300 mg/day) on fecal fat, FA and BA composition (Bendsen et al. 2008). Dairy Ca

increased fecal fat excretion. Fecal excretion of SFA, MUFA and PUFA were all increased with the greatest effects seen for MUFA. Fecal BA excretion was unaffected by higher Ca from dairy. Short-term high dietary Ca intake (1800 mg/day) was shown to increase fecal fat excretion by ~2.5-fold (Jacobsen et al. 2005). Ca supplementation of 2000 or 3000 mg/day for 16 weeks in individuals with a history of resected adenocarcinoma of the colon did not affect total fecal BA excretion (Lupton et al. 1996).

A study in female Wister rats showed that increasing dietary Ca increased fecal FA excretion, but did not affect total BA excretion (Govers & Meer 1993). Analysis of serum NS in obese Zucker rats fed diets containing 0.2, 0.8 or 2.1% Ca suggested that higher Ca increased intestinal cholesterol absorption and endogenous cholesterol synthesis (Vaskonen et al. 2002). The authors proposed that the lower serum LDL-C in the rats fed higher Ca is due to Ca-induced increase in the conversion of cholesterol to BA. Results from a study with ovariectomized hamsters fed 0, 2, 6 or 8 g of Ca per kg diet showed that the highest Ca dose increased fecal total NS and total BA excretion compared to the lowest Ca dose (Ma et al. 2011). Since differences in plasma lipids were seen amoung other diet groups would suggest that other mechanisms, other than effects of Ca on NS and BA absorption, may account for the chnages in blood lipids.

Human and animal studies have reported inconsistent results regarding the effects of dietary Ca on blood lipids and absorption of FA, NS and BA. Thus, more research on the effects of dietary Ca across a range of doses on serum lipids and absorption of FA, NS and BA is warranted.

2. Rationale and Objectives

CVD is the leading cause of mortality worldwide. Ca intakes may modify CVD risk by affecting known risk factors including body composition and blood lipids, but the topic is still controversial. Ca may modify risk through multiple mechanisms that may be dependent on Ca dose and overall diet (e.g., fat content and composition). To better understand the relationship between Ca intake and CVD risk factors, the effects of dietary Ca above and below nutrient requirements on body composition and blood lipids was examined in rats fed a high-fat, high-energy diet. FA digestibility and excretion of NS and BA were measured to gain insight into the possible underlying mechanisms driving changes in blood lipids. A better comprehension of dietary factors that impact body composition and lipid metabolism such as Ca is important for developing nutritional strategies to reduce the prevalence of chronic diseases such as CVD.

3. Materials and Methods

3.1 Diets and animal protocol

A schematic illustration of the study design is shown in Figure 8. One hundred and fifty male Sprague-Dawley CD rats (Charles River Canada, St. Constance, QC, Canada) at 42 days of age were assigned to 1 of 5 high-fat, high-energy diets (Dyets, Inc., Bethlehem, PA, USA) containing different amounts of Ca (0.75Ca, 0.86 ± 0.05; 2Ca, 2.26 ± 0.02; 5Ca, 5.55 ± 0.08; 10Ca,11.03 ± 0.17; 20Ca, 21.79 ± 0.15 g Ca/kg diet). Rats were assigned to diet groups (n=30/diet group) based on initial body weight to ensure that mean body weights were similar for each diet group at the start of the study. Compositions and energy densities of the diets are shown in Table 3. Diets were formulated using the AIN-93G mineral mix (Reeves et al. 1993) without Ca. Ca was added to the diets as Ca carbonate. Diets were adapted versions of the Research Diets, Inc. D12266B diet used previously to induce obesity in rats (Bertinato et al. 2014; Bertinato et al. 2016). For more accurate measurement of food consumption, diets were pelleted.

Rats were housed singly in solid-bottom cages with a wire grill insert and held in vented racks. Rats were put on a 12:12-hour light-dark cycle and had free access to food and demineralised water throughout the study. On a weekly basis, food consumption and body weight were measured. Body composition was measured using magnetic resonance imaging (EchoMRI-4in1™ system, EchoMRI, Houston, TX, USA) at the beginning (Day 0), middle (Day 35) and end (Day 70) of the study. Feces were collected daily for 7 consecutive days during weeks 3 and 8 of the study. Feces were immediately frozen each day after collection. Feces were freeze-dried and weighed prior to analysis.

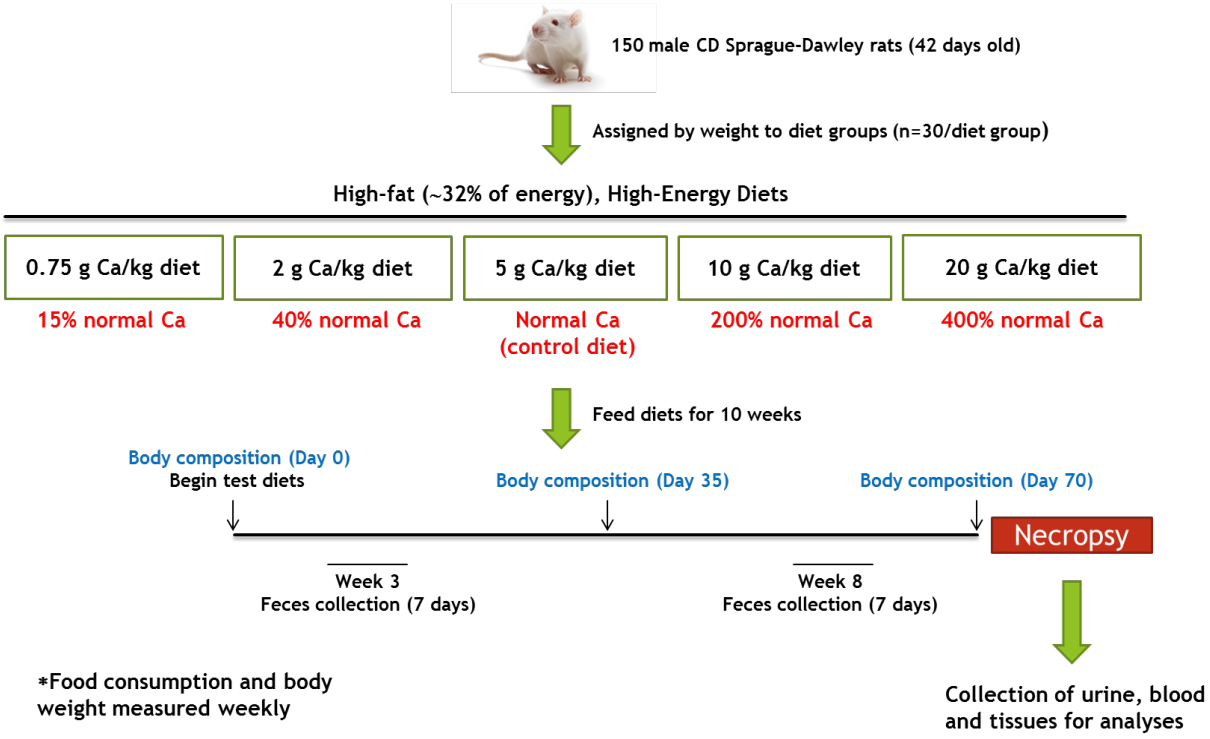


Figure 8. Study design.

Table 3. Diet compositions.

Component	Diets				
	0.75Ca	2Ca	5Ca	10Ca	20Ca
Fixed ingredients (g/kg) [1]	709	709	709	709	709
Sucrose (g/kg)	288.227	285.105	277.612	265.125	240.15
Energy density (kcal/kg) [2]	4437 (18.57)	4425 (18.52)	4395 (18.40)	4345 (18.19)	4245 (17.77)
CHO (% of energy)	51.5	51.4	51.0	50.5	49.3
Lipid (% of energy)	32.9	33.0	33.2	33.6	34.4
Protein (% of energy)	15.6	15.6	15.7	15.9	16.3
Ca carbonate (g/kg)	1.873	4.995	12.488	24.975	49.95
Ca concentration (g/kg WW) [3]	0.789 ± 0.048^{e}	2.09 ± 0.01^{d}	5.08 ± 0.07^{c}	10.1 ± 0.1^{b}	20.1 ± 0.1^{a}

[1] Fixed ingredients (g/kg): cornstarch, 202.191; casein, 190; corn oil, 118; dyetrose, 75; anhydrous milkfat, 44.2; AIN-93G mineral mix without Ca (No.: 213019), 35; cellulose (microcrystalline), 30; AIN-93G vitamin mix (No.: 310025), 10; L-cystine, 3; choline bitartrate, 2.5; ethoxyquin, 0.009. [2] Energy density in MJ/kg is shown in parentheses. [3] Analyzed concentrations. Values are means ± SD, n = 5. Values in the row without a common superscript letter differ, $p < 0.05$. Ca, calcium; CHO, carbohydrate; WW, wet weight.

After 10 weeks of feeding the diets rats were fasted overnight (12 hours) in metabolic cages for collection of urine and then killed the following morning by exsanguination under general isoflurane anesthesia. From the abdominal aorta, using a syringe, blood was collected and dispensed into blood tubes for isolation of serum (Trace Element Serum tube, Thermo Fisher Scientific, Ottawa, ON, Canada) and plasma (BD vacutainer K_2EDTA, Thermo Fisher Scientific). Liver was extracted, weighed and a section of the left lateral lobe was removed and snap-frozen in liquid nitrogen for gene expression experiments. The rest of the liver was frozen on dry ice. Inguinal, retroperitoneal plus perirenal, mesenteric and epididymal adipose depots were extracted and weighed. Tissues, feces, urine and serum/plasma samples were stored at −80°C until analysis. During the study 1 rat in the 0.75Ca group and 2 rats in the 2Ca group died unexpectedly without prior symptoms. Results from these rats are not reported. The experimental protocol was approved by the Health Products and Food Branch Animal Care Committee of Health Canada (Protocol No.: 2015-010).

3.2 Mineral analyses

Diet samples (~0.5 g) were weighed in quartz beakers and dried overnight in an Isotemp®oven (Thermo Fisher Scientific) at 100^{o}C. Samples were ashed using a combination of dry ashing using a Thermo Scientific Lindberg/Blue MTM box furnace (Thermo Fisher Scientific) and wet ashed using concentrated trace metal grade nitric acid (Thermo Fisher Scientific). Ashes were solubilized in dilute nitric acid. Solubilized ashes and urine samples were analyzed for mineral concentrations using a radial view 700 Series inductively coupled plasma optical emission spectrometer (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Operating conditions have been described previously (Bertinato et al. 2016). Standard calibration curves were prepared using the CALEDON-88 multi-element standard (Inorganic Ventures,

Christiansburg, VA, USA) and analytical precision was verified using National Institute of Standards and Technology traceable reference material™ (SCP Science, Baie D'Urfé, QC, Canada).

3.3 Assays

Plasma insulin and glucose concentrations were measure using the Rat Ultrasensitive Insulin ELISA (80-INSRTU-E01, Alpco Diagnostics, Salem, NH, USA) and Glucose Colorimetric Assay Kit (10009582, Cayman Chemical, Ann Arbor, MI, USA), respectively. PTH concentration was measured using the Rat BioActive Intact PTH ELISA Kit (60-2700, Immutopics, Inc., San Clemente, CA, USA). Serum lipids and urine creatinine were measured using the ABX Pentra 400 chemistry analyser (HORIBA Instruments Inc., Irvine, California, USA).

3.4 Measurement of total lipids and FA in feces and diets

Total lipids were extracted from ground, freeze-dried feces and diet samples using an improved Bligh and Dyer extraction procedure (Jensen 2008) with modifications. Briefly, ~100 mg of sample (weighed accurately) was hydrolyzed with 1 mL of 3 M HCL. To the samples, 2.25 mL of methanol:chloroform (2:1, v/v) and 0.25 mL (0.512 mg) of 13:0 internal standard (Nu-Check Prep, Waterville, MN, USA) was added. After addition of 2 mL of chloroform and 1 mL of water, the samples were vortexed, centrifuged (5 minutes, 2000 × g) and the bottom chloroform layer was collected into a pre-weighted glass tube. The chloroform was evaporated under nitrogen and the tube reweighed to obtain the weight of total lipids.

For determination of FA concentrations, total lipids were dissolved in 1 mL of toluene and converted into fatty acid methyl esters (FAME) by adding 0.5 mL of methanol and 0.5 mL of

borontrifluoride (in 14% methanol) and heating for 1 hour at 105°C. FAME were recovered by adding 2 mL of water and 2 mL of hexane to the sample, mixing and collecting the hexane layer. The hexane was dried with anhydrous sodium sulphate for a minimum of 20 minutes and the hexane was then transferred to a new tube and evaporated under nitrogen. The FAME were re-solubilised in 1 mL of hexane and 1 ul was analyzed by gas chromatography (Agilent 6890N system with an auto injector; Agilent, Santa Clara, CA, USA). The gas chromatograph was fitted with a flame ionization detector and a 100-m x 0.25-mm capillary column (SP-2560, Sigma Aldrich, Oakville, ON, Canada). The initial column oven temperature was 180^{o}C, followed by 2 ramps of 32 minutes (215^{o}C) and 65 minutes (240^{o}C). The injector and the detector temperatures were 250^{o}C. Ultra-high-purity hydrogen was used as the carrier gas at a flow rate of 0.8 mL/minute. Chromatographic peaks were identified by comparison with known fatty acid methyl ester standards (Nu-Chek Prep, Waterville, MN, USA and Sigma Aldrich). Concentrations of FA were determined by comparison to the internal standard. FA representing less than 0.05% of total FA were excluded from further analysis.

3.5 Measurement of lipids in liver

Total lipids were extracted from a large portion of the liver comprising all 4 lobes missing a small section of the left lateral lobe. The liver was ground with a meat grinder into a homogenous slurry and ~300 mg of slurry (weighed accurately) was homogenized in 5 mL of chloroform:methanol (2:1, v/v). The samples were incubated overnight on a shaker, centrifuged (10 minutes, 2000 × g) and the supernatant was collected. Sodium chloride (0.9%, w/v) was added to the supernatant and the organic solvent layer was recovered and evaporated under nitrogen to obtain the weight of total lipids. For determination of TC, FC and TG concentrations,

total lipids were extracted from a ~300 mg section (weighed accurately) of the left lateral lobe as described above. Total lipids were weighed and re-suspended in 1.5 mL of 10% Triton X-100 in isopropanol. TC, FC and TG were measured using Wako Cholesterol E (999-02601, Wako Chemicals, Richmond, VA, USA), Free Cholesterol E (993-02501, Wako Chemicals) and L-Type Triglyceride M (Wako Chemicals) kits, respectively.

3.6 Measurement of NS and BA

NS and BA were analysed following previously published methods (Czubayko et al. 1991; Chan et al. 1999) with some modifications. Approximately 100 mg of freeze-dried feces or diet (cholesterol measurement) were weighed accurately and 3 mL of 1N NaOH (in 90% ethanol) was added to the sample. Then, 0.25 mL of 5α-cholestane (0.110 mg) (Sigma Aldrich) and hyodeoxycholic acid (0.155 mg) (Sigma Aldrich) were added as internal standards for quantification of NS and BA, respectively. Samples were refluxed under nitrogen for 1 hour, cooled and 1 mL of water and 5 mL of petroleum ether were added. Samples were mixed and centrifuged (2 minutes, 1000 × g). The petroleum ether and aqueous layers containing NS and BA, respectively, were further processed. The petroleum ether layer was collected, dried with sodium sulphate, transferred to a new tube and evaporated to dryness under nitrogen. NS were derivitized by adding 0.5 mL of TMSI + pyridine (1:4, v/v) (Cat No.: 92718, Sigma Aldrich) and incubating at 60°C for 1 hour. Reagents were evaporated and derivitized NS were solubilized in 0.25 mL of hexane, centrifuged (2 minutes, 1000 × g) and the supernatant subjected to gas chromatography. For the extraction of BA, 0.5 mL of 10N NaOH was added to the aqueous layer, the samples were heated at 120°C for 3 hours, cooled and the sample was acidified by

adding 1 mL of concentrated HCL. Then, 7 mL of chloroform:methanol (2:1, v/v) was added, samples were mixed, centrifuged (2 minutes, 1000 × g) and the chloroform layer was collected. The chloroform was dried with sodium sulphate, transferred to a new tube and evaporated. BA were methylated by adding 1 mL of dried methanol, 1 mL of dimethoxypropane, 20 µl of concentrated HCL and incubating at room temperature for 2 hours in the dark. Reagents were evaporated and methylated BA were derivitized using TMSI + pyridine and subjected to gas chromatography as described for the NS.

Gas chromatography was performed using a 30-m x 0.25-mm capillary column (DB-1701, Agilent). The injector and the detector temperatures were 300^{o}C. NS were analyzed on an isothermal run at 270^{o}C. For BA, the initial oven temperature was 240^{o}C, followed by 2 ramps of 10 minutes at 275^{o}C and 42 minutes at 280^{o}C. NS and BA peaks were identified by comparing with known standards (Sigma Aldrich and Steraloids, Newport, RI, USA). Concentrations were determined by comparison with peaks of the corresponding internal standard.

3.7 QPCR

Total liver RNA was isolated from the left lateral lobe, purified and DNase I treated using the RNeasy Mini kits (Qiagen, Mississauga, ON, Canada). RNA was quantified using a NanoDrop Spectrophotometer (ThermoScientific, Wilmington, DE, USA) and the integrity of each sample was verified by agarose gel electrophoresis. Two µg of total RNA was reverse transcribed using random primers using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). QPCR was performed on a ViiA7 Quantitative PCR System (Applied Biosystems by Life Technologies, Austin, TX, USA) using TaqMan reagents and TaqMan gene expression assays for Cyp7a1 (Rn00564065_m1), Ldlr (Rn00598442_m1),

Hmgcr (Rn00565598_m1), Hmgcs1 (Rn01493959_m1), Fasn (Rn00569117_m1), Cpt2 (Rn00563995_m1), Acat2 (Rn01526241_g1), and 18s (Rn03928990_g1) (Applied Biosystems by Life Technologies). For each experiment, no template and no reverse transcriptase negative controls were included. The amounts of each gene-of-interest were determined using the standard curve method and normalized to 18s expression. Normalized values were calibrated to the 5Ca group (mean set as 1.00).

3.8 Calculations

Apparent digestibility of total lipids and individual FA was calculated from amounts consumed from the diet and amounts excreted in feces using the formula:

$$\text{Digestibility (\%)} = (\text{Total consumed} - \text{Total excreted})\,(\text{Total consumed})^{-1} \times 100$$

Cholesterol retained (CR) was calculated from amount of cholesterol consumed from the diet and amounts of NS (animal) and BA excreted in feces using the formula:

$$\text{CR} = \text{Cholesterol consumed} - \text{NS (animal) excreted} - \text{BA excreted}$$

3.9 Statistical analyses

Results are reported as means ± SD. Differences in means were determined by one-way ANOVA. When overall results were significant the Holm-Sidak post-hoc test was used to determine which means differed. Mixed-design ANOVA was used for analysis of parameters measured at multiple time points to determine the effects and interaction of diet and time. For time points with a significant diet effect differences among groups were determined by one-way ANOVA followed by the Holm-Sidak test. Homogeneity of variances was assessed using Levene's test. Data that showed unequal variances were transformed prior to analysis. When equality of variances could not be achieved the non-parametric Kruskal-Wallis ANOVA and multiple comparisons of mean ranks was used to determine differences among groups. Statistical significance was set at $p < 0.05$. Data were analyzed using Statistica 7.1 (StatSoft, Tulsa, OK, USA) and SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA, USA).

4. Results

4.1 Experimental diets

The objectives of this study was to examine the effects of different doses of dietary Ca below and above nutrient requirements on known risk factors for CVD including body composition, parameters of glucose metabolism and serum lipid concentrations. To better understand the possible underlying mechanisms by which dietary Ca may affect these risk factors, energy intake, energy efficiency, digestibility of fatty acids, fecal excretion of NS and BA and lipid metabolism in the liver were investigated.

The experimental diets were high-fat, high-energy diets. Energy densities of the diets ranged from 4245–4437 kcal/kg (Table 3). Amounts of ingredients added to the diets were similar for all 5 diets with the exception of Ca and sucrose. Different amounts of Ca were added to the diets as Ca carbonate at the expense of sucrose. Analysis of Ca in the diets confirmed the expected concentrations based on the amounts of Ca carbonate added. The normal amount of Ca for growing rats based on AIN-93G requirements is 5 g/kg diet (Reeves et al. 1993). The normal Ca diet contained 5.08 ± 0.07 g/kg diet by analysis (Table 3). The 2 low Ca diets contained 16% (0.75Ca) and 42% (2Ca) of normal Ca. The 2 high Ca diets contained 202% (10Ca) and 402% (20Ca) of normal Ca.

Carbohydrates contributed 49.3–51.5% of total energy of the diets and protein contributed 15.6–16.3% of total energy. The diets contained 32.9–34.4% of energy from fat. Anhydrous milk-fat and corn oil were used as fat sources. Milk-fat was used as a fat source because it comes from a ruminant source and contains TFA. This allowed investigation of the effects of dietary Ca on TFA digestibility.

Concentrations of cholesterol and FA in the diets were analyzed (Table 4). Cholesterol, total SFA, total MUFA, total PUFA, and total TFA concentrations were similar in the diets. Cholesterol concentration in the diets was 0.178 ± 0.003–0.184 ± 0.005 g/kg. Diet concentrations of total SFA, MUFA, PUFA and TFA were 42.9 ± 3.2–44.1± 1.9, 42.6 ± 3.9–43.8 ± 2.5, 67.6 ± 6.2–69 ± 4.0, and 2.46 ± 0.21–2.52 ± 0.16 g/kg, respectively. Total lipids were lower ($p < 0.05$) in the 20Ca diet compared to the 2Ca and 10Ca diets, but the magnitudes of the differences were small.

4.2 Effects of dietary Ca on urine mineral concentrations

Urine concentrations of Ca, Mg, P, K and Na (normalized to creatinine concentration) were measured. The concentrations of minerals in urine were different in rats fed diets with different amounts of Ca (Table 5). Ca concentration in urine was higher in rats fed the 20Ca diet compared to all other groups. Urine P concentration was lower in rats fed the 10Ca or 20Ca diets compared to rats fed the 5Ca diet. Rats fed the 0.75Ca or 2Ca diets had higher urine P concentrations compared to rats fed the 5Ca diet. Na concentration in urine was similar in rats fed the 5Ca diet compared to rats fed the low (0.75Ca and 2Ca) or high (10Ca and 20Ca) Ca diets. Rats fed the 0.75Ca diet had higher urine Na compared to rats fed the 2Ca or 10Ca diets. Mg and K concentrations in urine did not differ among groups.

Table 4. Analyzed lipid concentrations in diets.

Lipid (g/kg WW)	Diets				
	0.75Ca (n = 6)	2Ca (n = 6)	5Ca (n = 6)	10Ca (n = 6)	20Ca (n = 6)
10:0	0.465 ± 0.285	0.521 ± 0.253	0.442 ± 0.266	0.509 ± 0.235	0.429 ± 0.265
12:0	1.13 ± 0.09	1.20 ± 0.09	1.15 ± 0.08	1.21 ± 0.06	1.15 ± 0.10
14:0	4.52 ± 0.27	4.69 ± 0.16	4.57 ± 0.26	4.65 ± 0.07	4.63 ± 0.17
I15:0	0.081 ± 0.007	0.085 ± 0.003	0.081 ± 0.005	0.084 ± 0.003	0.084 ± 0.005
15:0	0.506 ± 0.033	0.514 ± 0.024	0.504 ± 0.029	0.512 ± 0.012	0.504 ± 0.027
I16:0	0.107 ± 0.010	0.109 ± 0.005	0.107 ± 0.007	0.109 ± 0.003	0.108 ± 0.005
16:0	27.8 ± 2.4	28.5 ± 1.5	27.8 ± 2.3	28.0 ± 0.7	28.2 ± 1.6
I17:0	0.151 ± 0.013	0.154 ± 0.010	0.152 ± 0.011	0.153 ± 0.005	0.152 ± 0.009
17:0	0.353 ± 0.031	0.365 ± 0.022	0.358 ± 0.031	0.359 ± 0.011	0.362 ± 0.025
18:0	6.79 ± 0.59	6.95 ± 0.42	6.82 ± 0.61	6.86 ± 0.18	6.91 ± 0.41
I18:0	0.024 ± 0.004	0.026 ± 0.004	0.026 ± 0.004	0.025 ± 0.002	0.026 ± 0.004
20:0	0.522 ± 0.048	0.532 ± 0.032	0.522 ± 0.048	0.523 ± 0.014	0.529 ± 0.031
22:0	0.187 ± 0.016	0.193 ± 0.014	0.188 ± 0.017	0.191 ± 0.006	0.192 ± 0.012
23:0	0.047 ± 0.005	0.047 ± 0.005	0.048 ± 0.005	0.047 ± 0.002	0.049 ± 0.005
24:0	0.210 ± 0.020	0.209 ± 0.019	0.209 ± 0.021	0.208 ± 0.007	0.211 ± 0.015
Total SFA	42.9 ± 3.2	44.1 ± 1.9	42.9 ± 3.0	43.4 ± 0.7	43.5 ± 2.0
16:1 9c	0.738 ± 0.067	0.758 ± 0.037	0.740 ± 0.064	0.747 ± 0.021	0.748 ± 0.039
16:1 11c	0.020 ± 0.001	0.023 ± 0.004	0.021 ± 0.003	0.021 ± 0.003	0.022 ± 0.003
16:1 13c	0.060 ± 0.006	0.064 ± 0.007	0.062 ± 0.006	0.063 ± 0.003	0.062 ± 0.006
17:1 9c	0.024 ± 0.007	0.028 ± 0.006	0.027 ± 0.004	0.026 ± 0.003	0.029 ± 0.007
18:1 9c	40.3 ± 3.7	41.1 ± 2.4	40.0 ± 3.7	40.2 ± 1.1	40.7 ± 2.5
18:1 11c	1.02 ± 0.09	1.04 ± 0.06	1.02 ± 0.09	1.02 ± 0.03	1.03 ± 0.06
18:1 12c	0.173 ± 0.019	0.188 ± 0.012	0.181 ± 0.018	0.182 ± 0.007	0.184 ± 0.010
18:1 13c	0.042 ± 0.006	0.047 ± 0.008	0.047 ± 0.004	0.044 ± 0.003	0.045 ± 0.003
18:1 14c	0.039 ± 0.004	0.039 ± 0.004	0.042 ± 0.008	0.038 ± 0.003	0.041 ± 0.002
18:1 15c	0.053 ± 0.007	0.056 ± 0.008	0.058 ± 0.005	0.053 ± 0.005	0.058 ± 0.004
Total 18:1 cis	41.6 ± 3.9	42.5 ± 2.4	41.4 ± 3.8	41.6 ± 1.2	42.1 ± 2.5
20:1 11c	0.360 ± 0.035	0.368 ± 0.021	0.358 ± 0.033	0.361 ± 0.010	0.364 ± 0.022
22:1 13c	0.026 ± 0.002^{b}	0.028 ± 0.003^{ab}	0.032 ± 0.002^{a}	0.026 ± 0.002^{b}	0.025 ± 0.002^{b}
24:1 15c	0.016 ± 0.003	0.016 ± 0.005	0.015 ± 0.001	0.017 ± 0.001	0.015 ± 0.003
Total MUFA	42.9 ± 4.0	43.8 ± 2.5	42.6 ± 3.9	42.8 ± 1.2	43.4 ± 2.6
18:2 9c, 11t	0.125 ± 0.013	0.126 ± 0.006	0.126 ± 0.011	0.132 ± 0.004	0.128 ± 0.008
18:2 9t, 11t	0.098 ± 0.012	0.102 ± 0.010	0.100 ± 0.010	0.101 ± 0.007	0.102 ± 0.010
18:2 10t, 12c	0.018 ± 0.005	0.018 ± 0.003	0.019 ± 0.003	0.020 ± 0.003	0.020 ± 0.002

18:2 n-6	66.6 ± 6.2	67.9 ± 3.9	65.9 ± 6.1	66.2 ± 1.9	67.1 ± 4.0
20:2 n-6	0.064 ± 0.007	0.067 ± 0.008	0.063 ± 0.007	0.066 ± 0.003	0.067 ± 0.007
20:3 n-6	0.060 ± 0.006	0.061 ± 0.004	0.060 ± 0.007	0.062 ± 0.002	0.060 ± 0.006
20:4 n-6	0.065 ± 0.007	0.067 ± 0.006	0.065 ± 0.008	0.065 ± 0.002	0.066 ± 0.006
22:4 n-6	0.021 ± 0.004	0.022 ± 0.003	0.021 ± 0.002	0.022 ± 0.003	0.019 ± 0.003
Total n-6 PUFA	66.8 ± 6.3	68.1 ± 3.9	66.1 ± 6.1	66.4 ± 1.9	67.3 ± 4.1
18:3 n-3	1.40 ± 0.13	1.43 ± 0.08	1.39 ± 0.13	1.40 ± 0.04	1.41 ± 0.09
22:5 n-3	0.042 ± 0.008	0.045 ± 0.008	0.040 ± 0.004	0.043 ± 0.006	0.044 ± 0.007
Total n-3 PUFA	1.44 ± 0.13	1.47 ± 0.09	1.43 ± 0.13	1.44 ± 0.05	1.46 ± 0.09
Total PUFA	68.3 ± 6.4	69.5 ± 4.0	67.6 ± 6.2	67.9 ± 2.0	68.8 ± 4.1
18:1 (6t-8t)	0.109 ± 0.010	0.108 ± 0.009	0.106 ± 0.011	0.107 ± 0.007	0.105 ± 0.008
18:1 9t	0.158 ± 0.012	0.164 ± 0.011	0.162 ± 0.013	0.161 ± 0.004	0.160 ± 0.007
18:1 10t	0.257 ± 0.020	0.268 ± 0.020	0.261 ± 0.022	0.266 ± 0.011	0.268 ± 0.014
18:1 11t	0.471 ± 0.037	0.480 ± 0.034	0.476 ± 0.042	0.478 ± 0.016	0.479 ± 0.034
18:1 12t	0.163 ± 0.012	0.168 ± 0.012	0.167 ± 0.015	0.168 ± 0.005	0.166 ± 0.009
18:1 (13t+14t)	0.385 ± 0.031	0.400 ± 0.027	0.395 ± 0.039	0.391 ± 0.010	0.394 ± 0.023
18:1 16t	0.154 ± 0.015	0.162 ± 0.011	0.161 ± 0.013	0.159 ± 0.005	0.159 ± 0.009
Total 18:1 TFA	1.70 ± 0.13	1.75 ± 0.11	1.73 ± 0.15	1.73 ± 0.05	1.73 ± 0.10
18:2 9t, 12t	0.108 ± 0.008	0.110 ± 0.007	0.109 ± 0.011	0.109 ± 0.008	0.109 ± 0.011
18:2 9c, 12t	0.328 ± 0.036	0.331 ± 0.022	0.320 ± 0.026	0.324 ± 0.012	0.328 ± 0.023
18:2 9t, 12c	0.224 ± 0.023	0.221 ± 0.016	0.222 ± 0.019	0.217 ± 0.014	0.221 ± 0.018
Total 18:2 TFA	0.660 ± 0.065	0.663 ± 0.044	0.651 ± 0.055	0.650 ± 0.031	0.658 ± 0.050
18:3 9t, 12c, 15c	0.106 ± 0.013	0.110 ± 0.006	0.106 ± 0.008	0.109 ± 0.005	0.109 ± 0.009
Total TFA	2.46 ± 0.21	2.52 ± 0.16	2.48 ± 0.21	2.49 ± 0.08	2.50 ± 0.16
Cholesterol [1]	0.178 ± 0.003	0.184 ± 0.005	0.181 ± 0.005	0.182 ± 0.002	0.181 ± 0.002
Total lipids	166 ± 5^{ab}	170 ± 2^{a}	163 ± 6^{ab}	167 ± 3^{a}	159 ± 4^{b}

Values are means ± SD. Values in a row without a common superscript letter differ, $p < 0.05$. [1] n = 3. CLA, conjugated linoleic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TFA, trans fatty acids; WW, wet weight.

Table 5. Urine mineral concentrations.

Parameter	Diet groups				
	0.75Ca	2Ca	5Ca	10Ca	20Ca
Urine Ca (mg/g Cr)	11 ± 3[b]	12 ± 2[b]	12 ± 4[b]	16 ± 21[b]	82 ± 94[a]
Urine Mg (mg/g Cr)	93 ± 41	109 ± 33	93 ± 39	84 ± 36	108 ± 32
Urine P (mg/g Cr)	1315 ± 334[a]	1368 ± 206[a]	1072 ± 282[b]	817 ± 334[c]	287 ± 339[d]
Urine K (mg/g Cr)	2935 ± 685	2780 ± 391	2727 ± 576	2774 ± 519	2619 ± 623
Urine Na (mg/g Cr)	320 ± 188[a]	191 ± 97[b]	248 ± 153[ab]	195 ± 131[b]	224 ± 161[ab]

Values are means ± SD, n=27–30. Values in a row without a common superscript letter differ, $p < 0.05$. Ca, calcium; Cr, creatinine; K, potassium; Mg, magnesium; Na, sodium; P, phosphorus.

4.3 Effects of dietary Ca on body weight and body composition

Body weight did not differ among the 2Ca, 5Ca, 10Ca or 20Ca groups throughout the study (Figure 9). At Day 35 of the study body weight of the 0.75Ca group was lower than that of the 10Ca group. At the end of the study (Day 70) body weight was lower for the 0.75Ca group compared to all other groups.

The effect of dietary Ca on percentage lean mass, total lean mass, percentage fat mass and total fat mass was examined at baseline (Day 0), midway through the study (Day 35) and at the end of the study (Day 70) using magnetic resonance imaging (MRI). None of these parameters differed among groups at baseline (Table 6). At Day 35, the 0.75Ca group showed higher percentage lean mass compared to the 5Ca, 10Ca and 20Ca groups. At Day 70, the 0.75Ca group showed higher percentage lean mass compared to all other groups. Total lean mass did not differ among groups at Day 35 or Day 70. At Day 35, the 0.75Ca group had lower percentage fat mass compared to the 5Ca and 10Ca groups. At Day 70, the 0.75Ca group had lower percentage fat mass compared to the 5Ca, 10Ca and 20Ca groups. The 0.75Ca group had lower total fat mass compared to all other groups at Day 70.

The effect of dietary Ca on the weight of different fat depots was also examined. Weights of inguinal, retroperitoneal plus perirenal, mesenteric or epididymal fat depots did not differ among the 2Ca, 5Ca, 10Ca or 20Ca groups (Table 6). Weights of inguinal, mesenteric and epididymal fat depots were lower in the 0.75Ca group compared to all other groups. The weight of the retroperitoneal plus perirenal fat depot was lower in the 0.75Ca group compared to the 5Ca, 10Ca and 20Ca groups. Collectively, these results demonstrate that dietary Ca in the

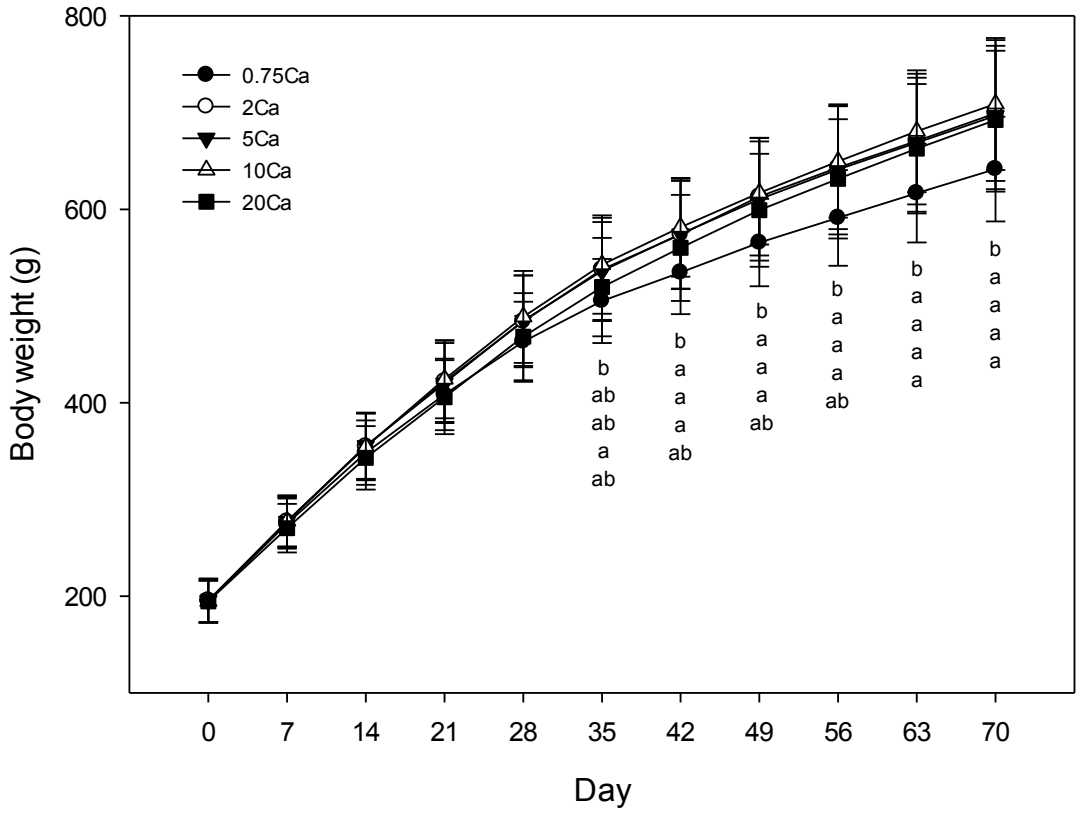


Figure 9. Body weights of rats. Results are presented as means ± SD, n = 28–30. Results were analyzed by mixed-design ANOVA to determine effects and interaction of diet and time. For time points with a significant ($p < 0.05$) diet effect, differences among diet groups were determined using one-way ANOVA followed by the Holm-Sidak post-hoc test. Diet groups without a common letter differ, $p < 0.05$. Letters correspond to diet groups 0.75Ca, 2Ca, 5Ca, 10Ca and 20Ca sequentially from top to bottom.

Table 6. Body composition of rats.

Parameter	Diet groups				
	0.75Ca (n = 29)	2Ca (n = 28)	5Ca (n = 30)	10Ca (n = 30)	20Ca (n = 30)
Lean (%) [1]					
Day 0	81.3 ± 2.1	81.3 ± 2.7	81.2 ± 2.3	81.1 ± 2.2	81.6 ± 2.5
Day 35	79.3 ± 3.8^{a}	76.5 ± 4.5^{ab}	75.3 ± 4.0^{b}	74.1 ± 3.8^{b}	76.1 ± 3.9^{b}
Day 70	76.3 ± 4.9^{a}	72.3 ± 4.6^{b}	70.9 ± 5.0^{b}	69.7 ± 3.9^{b}	70.1 ± 4.5^{b}
Lean (g) [1]					
Day 0	164 ± 16	163 ± 15	163 ± 15	163 ± 15	164 ± 16
Day 35	400 ± 28	410 ± 29	403 ± 24	401 ± 28	394 ± 29
Day 70	488 ± 38	504 ± 43	491 ± 35	493 ± 42	484 ± 39
Fat (%) [1]					
Day 0	11.9 ± 1.8	12.3 ± 2.0	12.3 ± 1.7	12.3 ± 1.8	11.8 ± 1.7
Day 35	17.3 ± 3.7^{b}	19.4 ± 4.3^{ab}	20.2 ± 4.1^{a}	21.3 ± 3.7^{a}	19.1 ± 3.6^{ab}
Day 70	19.8 ± 4.6^{b}	22.7 ± 4.5^{ab}	23.9 ± 4.9^{a}	25.1 ± 4.0^{a}	24.3 ± 4.5^{a}
Fat (g) [1]					
Day 0	24.3 ± 6.1	25.1 ± 6.7	25.1 ± 6.0	25.0 ± 6.2	24.0 ± 6.0
Day 35	88.3 ± 23.9^{b}	106 ± 32^{ab}	110 ± 32^{a}	117 ± 28^{a}	101 ± 26^{ab}
Day 70	128 ± 37^{b}	161 ± 42^{a}	169 ± 53^{a}	179 ± 39^{a}	170 ± 43^{a}
Ing fat (g) [2]	15.4 ± 5.7^{b}	21.2 ± 6.8^{a}	22.8 ± 10.8^{a}	24.9 ± 7.5^{a}	23.8 ± 8.2^{a}
Retro + Peri fat (g) [2]	22.4 ± 7.6^{b}	27.6 ± 8.5^{ab}	29.1 ± 8.3^{a}	31.8 ± 7.8^{a}	28.9 ± 7.9^{a}
Mes fat (g) [2]	7.0 ± 2.2^{b}	9.8 ± 3.1^{a}	10.1 ± 3.2^{a}	10.5 ± 2.6^{a}	10.8 ± 2.7^{a}
Epi fat (g) [2]	14.5 ± 3.8^{b}	18.9 ± 4.9^{a}	19.7 ± 5.1^{a}	20.7 ± 4.5^{a}	19.0 ± 4.8^{a}

Values are means ± SD. [1] Analyzed by mixed-design ANOVA to determine effects and interaction of diet and time. For time points with a significant ($p < 0.05$) diet effect, differences among diet groups were determined using one-way ANOVA followed by the Holm-Sidak post-hoc test. [2] Analyzed by one-way ANOVA and Holm-Sidak test. Values in a row without a common superscript letter differ, $p < 0.05$. Epi, epididymal; Ing, inguinal; Mes, mesenteric; Peri, perirenal; Retro, retroperitoneal.

range of ~2 to 20 g/kg diet did not affect body weight or body composition of the rats. A very low Ca diet containing approximately 16% of normal Ca requirements decreased body weight and fat mass of rats.

4.4 Effect of dietary Ca on food consumption, energy intake and energy efficiency

The effect of dietary Ca on food consumption, energy intake and energy efficiency was examined. Over the entire 10 week study (Overall), rats fed the 0.75Ca diet consumed less food compared to rats fed the 5Ca, 10Ca or 20Ca diets (Figure 10). Rats fed the 2Ca diet consumed less food compared to rats fed the 10Ca or 20Ca diets.

Given that the energy densities of the experimental diets differed slightly, energy intake of the rats was also examined. Overall energy intake was lower for the 0.75Ca group compared to the 5Ca, 10Ca or 20Ca groups (Figure 11A). Energy efficiency of the rats progressively declined over the course of the 10-week study as expected for growing rats of this age. Overall energy efficiency was higher for rats fed the 2Ca diet compared to rats fed the 0.75Ca or 20Ca diets (Figure 11B).

4.5 Effect of dietary Ca on serum lipid profile

The effect of dietary Ca on serum lipids was examined. Rats fed the high Ca diets (10Ca and 20Ca) had lower serum TC and LDL-C compared to rats fed the 5Ca diet (Figure 12A, B). Rats fed the low Ca diets (0.75Ca and 2Ca) had similar TC and LDL-C compared to rats fed the 5Ca diet. Serum HDL-C did not differ among groups (Figure 12C). Rats fed the 0.75Ca diet

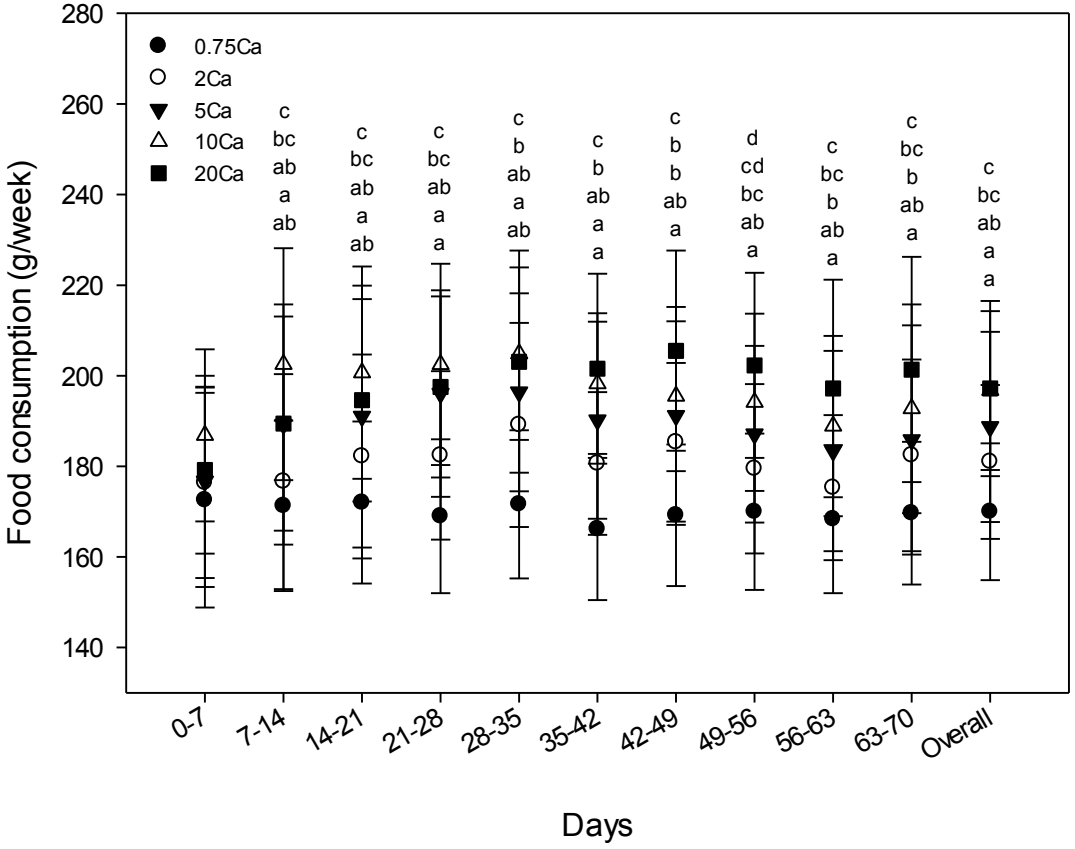
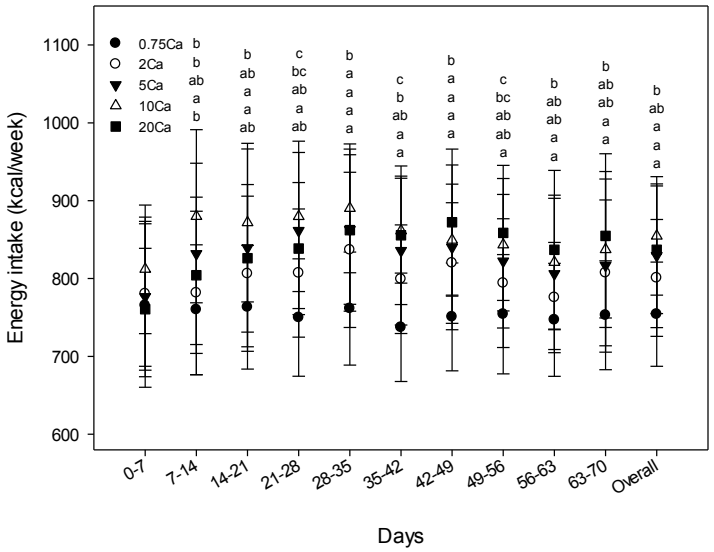


Figure 10. Food consumption of rats. Results are presented as means ± SD, n = 28–30. Results were analyzed by mixed-design ANOVA to determine effects and interaction of diet and time. For time points with a significant ($p < 0.05$) diet effect, differences among diet groups were determined using one-way ANOVA followed by the Holm-Sidak post-hoc test. Diet groups without a common letter differ, $p < 0.05$. Letters correspond to diet groups 0.75Ca, 2Ca, 5Ca, 10Ca and 20Ca sequentially from top to bottom. Overall food consumption was calculated by dividing the total food consumption for the entire study by the number of weeks of the study (10 weeks).

A



B

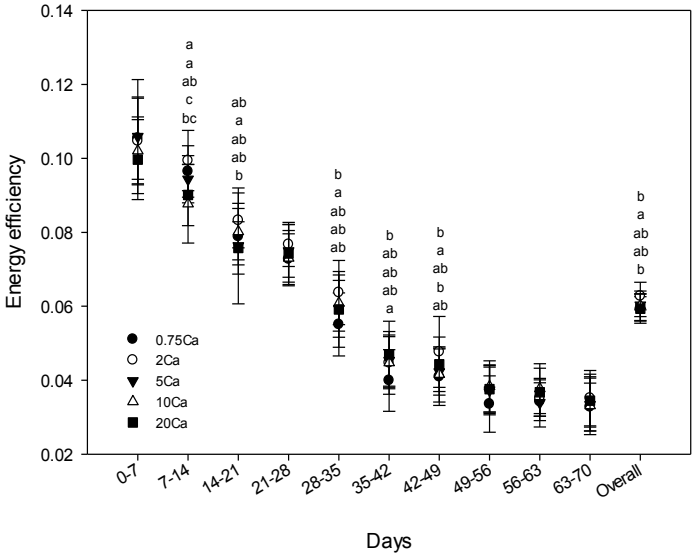


Figure 11. Energy intake (A) and energy efficiency (B) of rats. Results are presented as means ± SD, n = 28–30. Results were analyzed by mixed-design ANOVA to determine effects and interaction of diet and time. For time points with a significant ($p < 0.05$) diet effect, differences among diet groups were determined using one-way ANOVA followed by the Holm-Sidak post-hoc test. Diet groups without a common letter differ, $p < 0.05$. Letters correspond to diet groups 0.75Ca, 2Ca, 5Ca, 10Ca and 20Ca sequentially from top to bottom. Overall energy intake was calculated by dividing the total energy intake for the entire study by the number of weeks of the study (10 weeks). Energy efficiency = body weight gain (g/week)/energy intake (kcal/week). Overall energy efficiency was calculated by dividing the body weight gain for the entire study by the total energy intake.

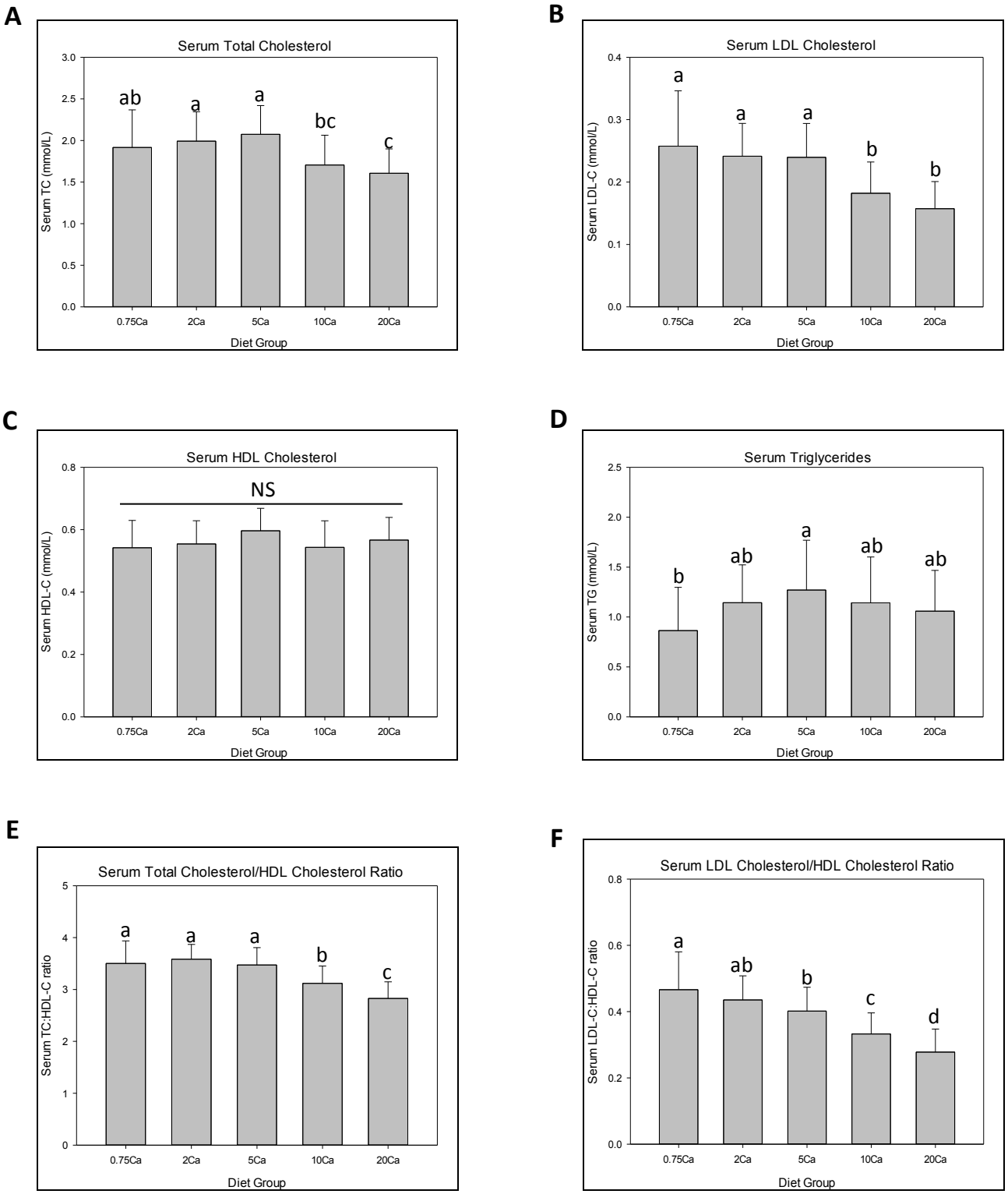


Figure 12. Serum lipid profile of rats. Fasting serum total cholesterol (A), LDL cholesterol (B), HDL cholesterol (C), triglycerides (D), total cholesterol: HDL cholesterol ratio (E), and LDL cholesterol:HDL cholesterol ratio (F) of rats. Results are presented as means ± SD, n = 28–30. Bars without a common letter differ, $p < 0.05$. NS, not significant.

had lower serum TG compared to rats fed the 5Ca diet (Figure 12D). Serum TG were similar in rats fed the 10Ca or 20Ca diets compared to rats fed the 5Ca diet. Serum TC:HDL-C ratio was lower for rats fed the 10Ca or 20Ca diets compared to rats fed the 5Ca diet (Figure 12E). Serum LDL-C:HDL-C ratio decreased in a dose-dependent manner in rats fed diets with higher Ca (Figure 12F).

4.6 Effect of dietary Ca on plasma glucose, insulin and parathyroid hormone (PTH) concentrations

The effect of dietary Ca on fasting plasma insulin, glucose and PTH concentrations was examined in the rats. Plasma insulin concentration was lower in rats fed the 0.75Ca diet compared to rats fed all other diets (Figure 13A). Plasma glucose concentrations did not differ among diet groups (Figure 13B). Rats fed the 20Ca diet had lower plasma PTH concentration compared to all other groups (Figure 13C).

4.7 Effect of dietary Ca on fecal weight

Figure 14A is a representative picture of total feces (freeze-dried) excreted over 2 days during week 3 of the study showing increased fecal bulk for rats fed higher Ca. All feces excreted over a 7-day period during week 3 or week 8 of the study was collected, freeze-dried and weighed. For rats fed the 2Ca, 5Ca, 10Ca or 20Ca diets, fecal weights during week 3 (Figure 14B) or week 8 (Figure 14C) increased in a dose-dependent manner in rats fed diets with higher Ca.

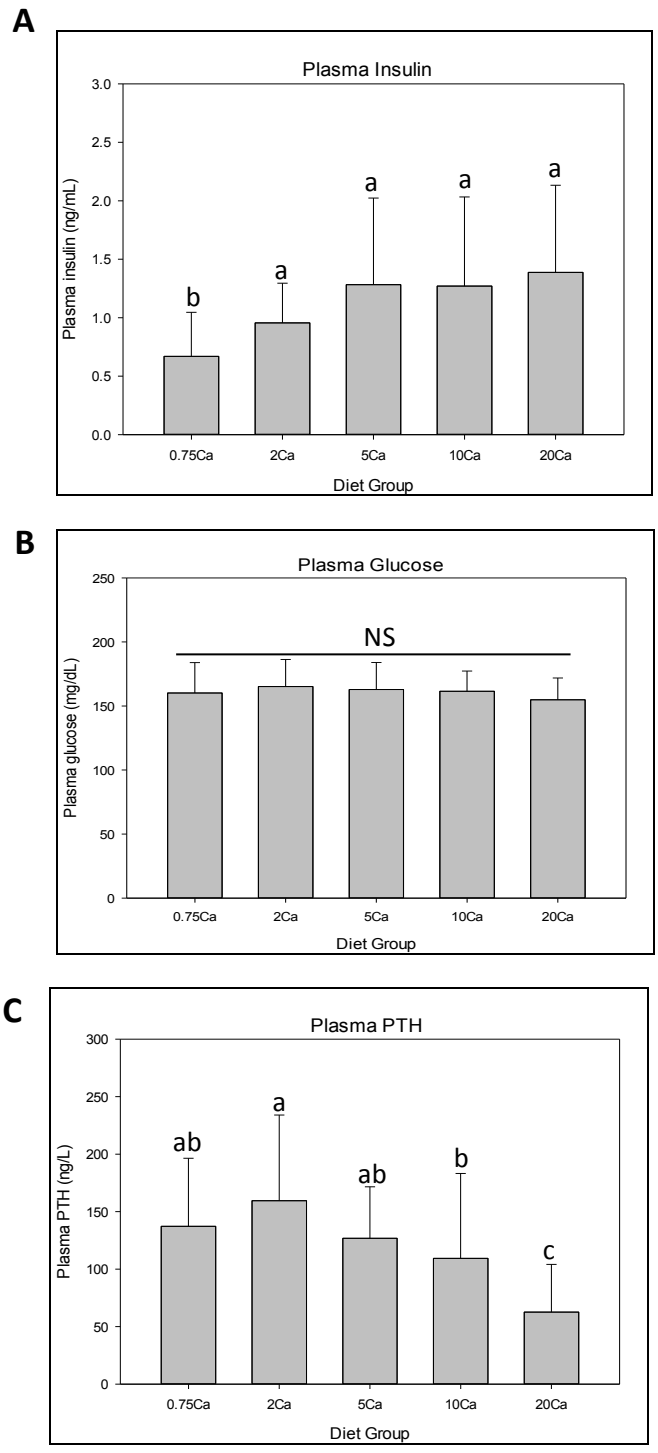


Figure 13. Plasma insulin (A), glucose (B), and PTH (C) concentrations of rats. Results are presented as means ± SD, n = 25–30. Bars without a common letter differ, $p < 0.05$. NS, not significant.

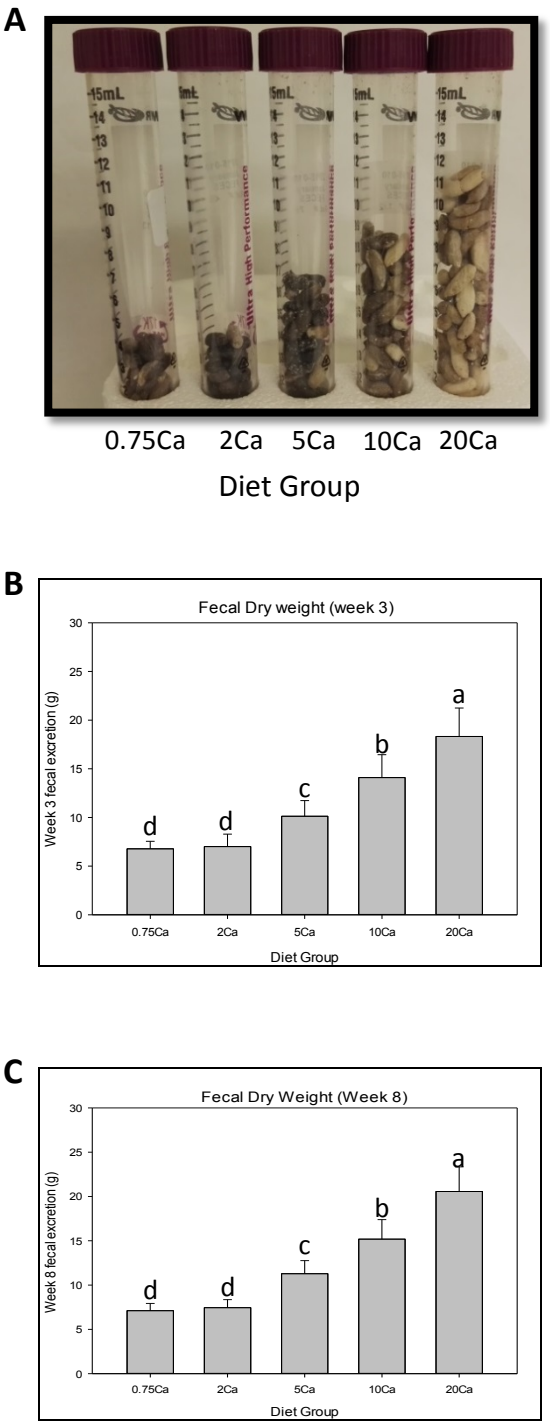


Figure 14. Fecal excretion of rats. Representative picture of the fecal excretion of rats over 2 days during week 3 of the study (A) and dry weight of 7 days of fecal excretion during week 3 (B) and week 8 (C) of the study. Results are presented as means ± SD, n = 28–30. Bars without a common letter differ, $p < 0.05$.

4.8 Effect of dietary Ca on liver weight and lipid concentrations

The effect of dietary Ca on liver weight (as a percentage of body weight) and total lipid, TC, FC and TG concentrations in the liver was examined. Rats fed the 2Ca diet had higher liver weight normalized to body weight compared to rats fed the 5Ca diet (Table 7). Rats fed the 10Ca or 20Ca diets had lower liver weight compared to rats fed the 5Ca diet. Total lipid concentration in the liver was higher for rats fed the 2Ca diet compared to rats fed the 10Ca or 20Ca diets. TC concentration in the liver was lower in rats fed the 20Ca diet compared to rats fed the 5Ca diet. Rats fed the low Ca diets (0.75Ca and 2Ca) had higher TC in the liver compared to rats fed the high Ca diets (10Ca and 20Ca). FC and TG concentrations in the liver did not differ among groups.

4.9 Effect of dietary Ca on fatty acid digestibility

The apparent digestibility of total lipids and FA was determined during week 3 of the study. Digestibility of total lipids was 98% in rats fed the low Ca diets, 93% in rats fed the 5Ca diet and 89% in rats fed the high Ca diets (Table 8). Dietary Ca affected the digestibility of all 4 FA classes. The largest effect was observed for TFA with a reduction from 98% in rats fed the low Ca diets to 42-46% in rats fed the high Ca diets. Digestibility of the primary natural (ruminant) TFA 18:1 11t was 92% in rats fed the low Ca diets and −144 to −120% in rats fed the high Ca diets. Digestibility of the most common industrial TFA 18:1 9t and 18:1 10t decreased from 99% in rats fed the low Ca diets to 85−86% in rats fed the high Ca diets. The digestibility of total conjugated linoleic acids (CLA) decreased from 98% to 44−49% in rats fed the low or

Table 7. Liver weight and lipid concentrations.

Parameter	Diet groups				
	0.75Ca (n = 29)	2Ca (n = 28)	5Ca (n = 30)	10Ca (n = 30)	20Ca (n = 30)
Liver weight (% BW)	2.91 ± 0.29^{ab}	3.04 ± 0.32^{a}	2.75 ± 0.29^{b}	2.45 ± 0.29^{c}	2.30 ± 0.19^{c}
Total lipids (mg/g)	114 ± 33^{ab}	137 ± 41^{a}	116 ± 35^{ab}	106 ± 34^{b}	101 ± 23^{b}
TC (mg/g)	5.02 ± 0.89^{a}	4.86 ± 1.11^{a}	4.45 ± 0.99^{ab}	3.91 ± 0.99^{bc}	3.74 ± 0.66^{c}
FC (mg/g)	2.16 ± 0.45	2.07 ± 0.37	2.01 ± 0.49	2.04 ± 0.41	1.91 ± 0.22
TG (mg/g)	48.3 ± 20.4	55.1 ± 25.7	51.1 ± 26.8	44.2 ± 27.4	39.1 ± 16.3

Values are means ± SD. Values in a row without a common superscript letter differ, $p < 0.05$. BW, body weight; FC, free cholesterol; TC, total cholesterol; TG, triglycerides.

Table 8. Apparent total lipid and FA digestibility during week 3 of the study.

Lipid	Diet groups				
	0.75Ca (n = 29)	2Ca (n = 28)	5Ca (n = 30)	10Ca (n = 30)	20Ca (n = 30)
			Digestibility (%)		
12:0	100 ± 0[a]	100 ± 0[a]	98 ± 1[b]	97 ± 1[c]	97 ± 1[c]
14:0	100 ± 0[a]	100 ± 0[a]	96 ± 1[b]	93 ± 2[c]	93 ± 2[bc]
I15:0	93 ± 1[b]	94 ± 2[a]	90 ± 2[c]	87 ± 4[d]	85 ± 2[e]
15:0	96 ± 1[a]	96 ± 1[a]	86 ± 3[b]	82 ± 4[c]	81 ± 3[c]
I16:0	95 ± 1[a]	95 ± 2[a]	88 ± 3[b]	82 ± 5[bc]	81 ± 4[c]
16:0	100 ± 0[a]	100 ± 0[a]	86 ± 4[b]	80 ± 6[bc]	80 ± 5[c]
I17:0	99 ± 0[a]	98 ± 1[a]	89 ± 3[b]	82 ± 5[c]	81 ± 4[c]
17:0	99 ± 0[a]	99 ± 0[a]	82 ± 5[b]	75 ± 6[b]	75 ± 6[b]
18:0	99 ± 0[a]	99 ± 0[a]	75 ± 7[b]	69 ± 8[b]	68 ± 7[b]
I18:0	85 ± 4[a]	85 ± 8[a]	37 ± 18[b]	12 ± 26[bc]	5 ± 16[c]
20:0	98 ± 0[a]	98 ± 1[a]	71 ± 7[b]	63 ± 9[b]	62 ± 8[b]
22:0	92 ± 2[a]	92 ± 2[a]	62 ± 8[b]	54 ± 10[bc]	51 ± 7[c]
23:0	86 ± 3[a]	86 ± 4[a]	60 ± 8[b]	52 ± 9[b]	51 ± 7[b]
24:0	86 ± 3[a]	85 ± 4[a]	43 ± 11[b]	32 ± 13[bc]	26 ± 9[c]
Total SFA	99 ± 0[a]	99 ± 0[a]	85 ± 4[b]	79 ± 5[b]	79 ± 5[b]
16:1 9c	100 ± 0[a]	100 ± 0[a]	99 ± 0[b]	97 ± 1[c]	97 ± 1[c]
16:1 11c	97 ± 1[a]	97 ± 1[a]	93 ± 2[b]	91 ± 3[bc]	90 ± 3[c]
16:1 13c	89 ± 3[b]	91 ± 2[a]	85 ± 3[c]	81 ± 4[d]	79 ± 4[d]
17:1 9c	73 ± 7[a]	76 ± 6[a]	60 ± 7[b]	56 ± 10[b]	58 ± 5[b]
18:1 9c	100 ± 0[a]	100 ± 0[a]	98 ± 1[b]	94 ± 3[c]	94 ± 3[c]
18:1 11c	99 ± 0[a]	99 ± 0[a]	95 ± 1[b]	89 ± 4[c]	89 ± 3[c]
18:1 12c	100 ± 0[a]	100 ± 0[a]	93 ± 4[b]	92 ± 3[b]	91 ± 3[b]
18:1 13c	98 ± 0[a]	98 ± 1[a]	88 ± 5[b]	84 ± 6[b]	82 ± 5[b]
18:1 14c	98 ± 0[a]	98 ± 1[a]	73 ± 7[b]	61 ± 11[b]	62 ± 7[b]
18:1 15c	100 ± 0[a]	100 ± 0[a]	89 ± 4[b]	84 ± 5[b]	85 ± 4[b]
Total 18:1 cis	100 ± 0[a]	100 ± 0[a]	98 ± 1[b]	94 ± 3[c]	94 ± 3[c]
20:1 11c	99 ± 0[a]	99 ± 0[a]	94 ± 2[b]	86 ± 5[c]	86 ± 5[c]
22:1 13c	93 ± 2[a]	94 ± 1[a]	68 ± 8[b]	43 ± 14[c]	30 ± 12[c]
24:1 15c	84 ± 3[a]	86 ± 4[a]	68 ± 7[b]	63 ± 8[bc]	50 ± 9[c]
Total MUFA	100 ± 0[a]	100 ± 0[a]	98 ± 1[b]	94 ± 3[c]	94 ± 3[c]
18:2 9c, 11t	99 ± 1[a]	98 ± 1[a]	93 ± 5[b]	47 ± 54[c]	43 ± 70[c]

18:2 9t, 11t	97 ± 2[a]	98 ± 2[a]	91 ± 4[b]	48 ± 44[c]	42 ± 80[c]
18:2 10t, 12c	96 ± 2[ab]	97 ± 1[a]	93 ± 2[b]	69 ± 29[c]	64 ± 59[c]
Total CLA	98 ± 1[a]	98 ± 1[a]	92 ± 4[b]	49 ± 47[c]	44 ± 72[c]
18:2 n-6	100 ± 0[a]	100 ± 0[a]	99 ± 0[b]	99 ± 1[c]	99 ± 1[c]
20:2 n-6	96 ± 1[a]	97 ± 1[a]	91 ± 2[b]	86 ± 5[c]	84 ± 5[c]
20:3 n-6	91 ± 4[b]	95 ± 3[a]	92 ± 4[b]	82 ± 9[c]	79 ± 8[c]
20:4 n-6	86 ± 5[a]	86 ± 7[a]	79 ± 7[a]	53 ± 18[b]	47 ± 16[b]
22:4 n-6	87 ± 3[b]	90 ± 2[a]	85 ± 4[b]	76 ± 7[c]	65 ± 9[d]
Total n-6 PUFA	100 ± 0[a]	100 ± 0[a]	99 ± 0[b]	99 ± 1[c]	98 ± 1[c]
18:3 n-3	99 ± 0[a]	99 ± 0[a]	97 ± 1[b]	93 ± 2[c]	93 ± 2[c]
22:5 n-3	98 ± 1[a]	99 ± 1[a]	98 ± 2[a]	95 ± 2[b]	94 ± 4[b]
Total n-3 PUFA	99 ± 0[a]	99 ± 0[a]	95 ± 1[b]	91 ± 3[c]	90 ± 2[c]
Total PUFA	100 ± 0[a]	100 ± 0[a]	99 ± 0[b]	98 ± 1[c]	98 ± 1[c]
18:1 (6t-8t)	100 ± 0[a]	99 ± 0[a]	82 ± 11[b]	87 ± 5[b]	86 ± 4[b]
18:1 9t	99 ± 0[a]	99 ± 0[a]	90 ± 4[b]	86 ± 5[b]	85 ± 4[b]
18:1 10t	99 ± 0[a]	99 ± 1[a]	88 ± 5[b]	86 ± 6[b]	85 ± 5[b]
18:1 11t	92 ± 4[a]	92 ± 4[a]	26 ± 45[b]	-144 ± 112[c]	-120 ± 96[c]
18:1 12t	99 ± 0[a]	99 ± 0[a]	91 ± 3[b]	86 ± 5[bc]	85 ± 4[c]
18:1 (13t+14t)	100 ± 0[a]	100 ± 0[a]	87 ± 4[b]	81 ± 6[b]	81 ± 5[b]
18:1 16t	100 ± 0[a]	100 ± 0[a]	87 ± 4[b]	81 ± 5[bc]	80 ± 5[c]
Total 18:1 TFA	97 ± 1[a]	97 ± 1[a]	71 ± 15[b]	21 ± 34[c]	27 ± 29[c]
18:2 9c, 12t	100 ± 0[a]	100 ± 0[a]	98 ± 1[b]	95 ± 2[c]	95 ± 2[c]
18:2 9t, 12c	98 ± 1[a]	97 ± 1[a]	89 ± 3[b]	85 ± 5[bc]	82 ± 5[c]
Total 18:2 TFA	99 ± 0[a]	99 ± 0[a]	95 ± 1[b]	92 ± 3[bc]	91 ± 2[c]
18:3 9t, 12c, 15c	100 ± 0[a]	100 ± 0[a]	95 ± 2[b]	87 ± 5[c]	87 ± 5[c]
Total TFA	98 ± 1[a]	98 ± 1[a]	78 ± 11[b]	42 ± 24[c]	46 ± 21[c]
Total lipids	98 ± 0[a]	98 ± 1[a]	93 ± 2[b]	89 ± 3[c]	89 ± 3[c]

Values are means ± SD. Values in a row without a common superscript letter differ, $p < 0.05$. CLA, conjugated linoleic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TFA, trans fatty acids.

High Ca diets, respectively. Comparable digestibility was observed for a naturally occurring isomer of CLA 18:2 9c, 11t.

Digestibility of total SFA decreased from 99% in rats fed the low Ca diets to 79–85% in rats fed the 5Ca or high Ca diets. In general, Ca had a greater effect on the digestibility of longer chain SFA. For SFA with a carbon chain length ≥ 20 the digestibility was 26–63% in rats fed the high Ca diets compared to 85–98% in rats fed the low Ca diets.

Compared to TFA or SFA, the digestibility of total MUFA and PUFA were less affected by the Ca content in the diets. Total MUFA digestibility decreased from 100% in rats fed the low Ca diets to 94% in rats fed the high Ca diets. The corresponding digestibility for total PUFA was 100% and 98%.

Dietary Ca had only a small effect on the digestibility of total n-6 PUFA or linoleic acid (18:2 n-6) as evidenced by a digestibility of ≥ 98% in rats fed the high Ca diets. Longer chain n-6 PUFA were more affected by dietary Ca with digestibility of 47–86% in rats fed the high Ca diets. Total n-3 PUFA digestibility was reduced from 99% in rats fed the low Ca diets to 90–91% in rats fed the high Ca diets. Comparable digestibility was observed for α-linolenic acid (18:3 n-3).

4.10 Effect of dietary Ca on NS and BA excretion

Fecal excretion of NS and BA was determined during week 8 of the study. Fecal excretion of cholesterol was higher in rats fed the 20Ca diet compared to all other groups (Table 9). Compared to the 5Ca group, excretion of total animal NS (cholesterol, coprostanol and cholestanol) was higher in rats fed the 20Ca diet and lower in rats fed the low Ca diets.

Table 9. Fecal excretion of NS and BA during week 8 of the study.

Parameter	Diet groups				
	0.75Ca (n = 9)	2Ca (n = 9)	5Ca (n = 9)	10Ca (n = 9)	20Ca (n = 9)
Fecal NS excretion (mg/wk)					
cholesterol	47.9 ± 9.1^{c}	56.2 ± 10.0^{bc}	51.3 ± 8.2^{bc}	65.0 ± 8.2^{b}	106 ± 38^{a}
coprostanol	7.19 ± 7.34^{b}	9.86 ± 6.63^{b}	38.4 ± 10.9^{a}	31.3 ± 11.8^{a}	15.5 ± 11.0^{b}
cholestanol	0.82 ± 0.24	0.97 ± 0.20	0.93 ± 0.17	0.99 ± 0.26	1.21 ± 0.59
Total animal NS excretion	55.9 ± 9.5^{c}	67.0 ± 9.7^{c}	90.7 ± 14.8^{b}	97.3 ± 12.3^{ab}	123 ± 34^{a}
β-sitosterol	83.1 ± 22.7^{b}	93.9 ± 17.9^{ab}	53.6 ± 14.0^{b}	72.8 ± 14.9^{b}	133 ± 65^{a}
stigmasterol	9.15 ± 2.12^{b}	10.2 ± 2.0^{ab}	6.54 ± 1.41^{b}	8.27 ± 1.39^{b}	14.3 ± 6.9^{a}
Total plant NS excretion	92.3 ± 24.8^{b}	104 ± 20^{ab}	60.2 ± 15.4^{b}	81.1 ± 16.3^{b}	147 ± 72^{a}
Fecal BA excretion (mg/wk)					
α-muricholic acid	2.99 ± 1.24^{b}	2.44 ± 1.00^{b}	3.11 ± 1.43^{b}	2.98 ± 1.06^{b}	6.53 ± 2.65$^{1, a}$
β-muricholic acid	16.7 ± 6.2	17.5 ± 10.6	14.6 ± 5.3	10.8 ± 3.8	23.0 ± 21.5^{1}
γ-muricholic acid	1.32 ± 0.55^{b}	0.84 ± 0.33^{b}	1.09 ± 0.47^{b}	1.27 ± 0.35^{b}	2.47 ± 1.21$^{1, a}$
cholic acid	3.76 ± 2.38^{a}	1.61 ± 1.25^{b}	0.65 ± 0.21^{b}	1.22 ± 0.69^{b}	3.53 ± 1.96$^{1, a}$
deoxycholic acid	4.93 ± 1.73^{b}	4.88 ± 1.98^{b}	13.3 ± 2.7^{a}	15.1 ± 5.2^{a}	17.8 ± 6.3$^{1, a}$
chenodeoxycholic acid	0.74 ± 0.52^{b}	0.67 ± 0.55^{b}	0.71 ± 0.25^{b}	1.07 ± 0.66^{ab}	1.87 ± 1.18$^{1, a}$
lithocholic acid	1.47 ± 0.51^{b}	1.22 ± 0.41^{b}	4.17 ± 1.63^{a}	4.47 ± 1.78^{a}	5.35 ± 2.33$^{1, a}$
Total BA excretion	31.9 ± 9.6^{b}	29.2 ± 11.7^{b}	37.5 ± 9.0^{b}	36.9 ± 8.9^{b}	60.6 ± 21.7$^{1, a}$
CR (mg/wk)	−56.7 ± 17.4^{a}	−61.6 ± 17.0^{a}	−93.0 ± 15.7^{b}	−97.8 ± 11.8^{b}	−142 ± 55$^{1, c}$

Values are means ± SD. Values in a row without a common superscript letter differ, $p < 0.05$. [1] n = 8. CR = Cholesterol intake − Neutral sterols (animal) excreted − Bile acids excreted. BA, bile acid; CR, cholesterol retained; NS, neutral sterol; wk, week.

Excretion of total plant NS (β-sitosterol and stigmasterol) was higher in rats fed the 20Ca diet compared to rats fed the 5Ca diet. Excretion of total BA was higher in the 20Ca group compared to all other groups. Cholesterol retained (calculated as the difference between cholesterol intake and the sum of total animal NS and total BA excreted) was lower for rats fed the 20Ca diet and higher for rats fed the low Ca diets compared to rats fed the 5Ca diet.

4.11 Effect of dietary Ca on expression of lipogenic genes in liver

Given the differences in lipid and TC concentrations in the liver of rats fed different amounts of Ca, the mRNA expressions of key lipid metabolism genes were measured in the liver. The expression of the *CYP7A1* gene involved in bile acid synthesis was similar among groups (Table 10). The expression of *LDLR* that functions in LDL clearance from the circulation was increased in rats fed diets with higher Ca. *HMGCR* and *HMGCS1* that function in cholesterol synthesis were also increased in a dose-dependent manner in rats fed diets with higher Ca. *FASN* that is involved in FA synthesis was decreased in rats fed the low Ca diets (0.75Ca and 2Ca) compared to rats fed the 5Ca diet or high Ca diets (10Ca and 20Ca). *CPT2* and *ACAT2* that function in FA oxidation and cholesterol esterification, respectively, were increased in a dose-dependent manner in rats fed diets with higher Ca. The observed changes in expression of lipogenic enzymes are consistent with a feedback response to the changes in lipid and cholesterol concentrations in the liver of rats fed different amounts of dietary Ca.

Table 10. Liver mRNA expression of lipogenic genes.

Gene	Diet groups				
	0.75Ca (n = 29)	2Ca (n = 28)	5Ca (n = 30)	10Ca (n = 30)	20Ca (n = 30)
CYP7A1	1.23 ± 1.37	0.89 ± 0.66	1.00 ± 0.94	1.35 ± 1.71	1.95 ± 2.07
LDLR	0.73 ± 0.39^{d}	0.70 ± 0.31^{d}	1.00 ± 0.50^{c}	1.42 ± 0.58^{b}	2.64 ± 1.28^{a}
HMGCR	0.55 ± 0.28^{d}	0.74 ± 0.42^{cd}	1.00 ± 0.58^{c}	1.71 ± 1.23^{b}	2.51 ± 1.20^{a}
HMGCS1	0.63 ± 0.37^{d}	0.75 ± 0.40^{cd}	1.00 ± 0.53^{c}	1.53 ± 0.66^{b}	3.40 ± 1.80^{a}
FASN	0.27 ± 0.16^{c}	0.46 ± 0.42^{b}	1.00 ± 1.29^{a}	0.93 ± 0.68^{a}	0.87 ± 0.61^{a}
CPT2	0.37 ± 0.19^{e}	0.59 ± 0.33^{d}	1.00 ± 0.39^{c}	1.61 ± 0.52^{b}	2.52 ± 0.91^{a}
ACAT2	0.61 ± 0.27^{d}	0.65 ± 0.31^{d}	1.00 ± 0.54^{c}	1.69 ± 0.72^{b}	3.71 ± 1.94^{a}

Relative mRNA expressions of lipid metabolism genes in liver (normalized to 18S rRNA). Results are presented as means ± SD. The mean of the 5Ca group (normal calcium) was arbitrarily set to 1. Values in a row without a common letter differ, $p < 0.05$.

5. Discussion

5.1 Ca intakes and CVD

Ca intakes at current recommendations and possibly higher may lower CVD risk by affecting know risk factors including body composition and serum lipids, but the topic remains controversial. Randomized controlled trials (RCT) and meta-analyses of RCT of Ca supplementation (with or without vitamin D) have reported a modest increase in risk of cardiovascular events, in particular myocardial infraction (Bolland et al. 2011). In a RCT by Bolland et al Ca supplementation of 1000 mg per day in postmenopausal women showed increased rates of cardiovascular events including sudden death, stroke and myocardial infraction (Bolland et al. 2008). In a meta-analysis by Bolland et al consumption of 500 mg or more of Ca supplement for at least 12 months showed increased risk of myocardial infraction (Bolland et al. 2010). The authors have proposed a mechanism to explain the effect of Ca supplementation and increased risk of CVD. Ca supplementation is associated with a transient increase in serum Ca concentration resulting in Ca deposition causing vascular calcification. Even though Ca supplementation may help prevent osteoporosis, it has been argued that it also increases the risk of calcification in the artery walls leading to serious health complications (Anderson et al. 2016).

Even though some studies have suggested that Ca supplementation may increase the risk of cardiovascular events, the totality of available evidence does not seem to support a causal inference between higher dietary Ca or Ca supplementation and increased risk of cardiovascular events (Waldman et al. 2015; Lappe & Heaney 2012). A recent updated systematic review and meta-analysis concluded that Ca intakes below the upper limit (2000–2500 mg/day) was not associated with increased risk of CVD events or mortality in healthy adults (Chung et al. 2016).

In a longitudinal cohort study with 6814 healthy volunteers, consumption of Ca from natural sources or supplements for l0 years showed that higher Ca intake from food was associated with reduced risk of atherosclerosis, while Ca supplementation was associated with increased risk of atherosclerosis (Anderson et al. 2016). These results suggest that if higher Ca intakes increase the risk for CVD disease and CVD events, it is likely from consumption of higher-dose Ca supplements and not from higher dietary (from food) Ca intakes.

At present, the effect of Ca intakes on CVD risk is unclear. The effects of Ca intakes on body composition and serum lipids (known risk factors for CVD) may depend not only on Ca dose but also overall diet such as fat content and composition. This study examined the effects of dietary Ca across a range of doses on body composition and serum lipids in rats fed a high-fat, high energy diet. To gain insight into the mechanisms accounting for changes in body composition and serum lipids energy intake, FA digestibility, lipid metabolism in the liver and fecal excretion of NS and BA were examined.

5.2 Dietary Ca alters serum PTH and urine mineral concentrations

CaSR in the parathyroid gland regulates Ca level in the blood. The receptors sense when there is a drop in serum Ca concentration. A drop in serum Ca leads to an increase in the production of PTH by the parathyroid gland. Higher circulating PTH stimulates 1α-hydroxylase activity in the kidney which converts 25-hydroxyvitamin D to the active form 1,25 dihydroxyvitamin D (also called calcitriol). This increases intestinal Ca absorption and stimulates release of Ca from bone. There is also inhibition of renal Ca excretion. The overall effect is a rise in serum Ca concentration. Under excess Ca consumption an increase in serum Ca

decreases PTH secretion from the parathyroid gland. This results in elevated urinary Ca excretion and a decrease in intestinal Ca absorption and resorption from bone (Ross et al. 2011).

The results from this study showed that rats fed different amounts of dietary Ca had changes in plasma PTH and urine minerals. Rats fed the highest Ca diet (20Ca) had lower plasma PTH and higher urine Ca concentrations compared to other groups. Also, serum Ca concentrations in rats fed the 20Ca diet were higher compared to rats fed the low Ca diets (0.75Ca and 2Ca) (data not shown). These results are consistent with the decrease in PTH secretion in response to higher serum Ca (Digirolamo et al. 2012). Lower circulating PTH concentrations attenuate increases in serum Ca by increasing urinary Ca excretion and decreasing reabsorption of Ca from bone (Ross et al. 2011).

Metabolism of both P and Mg are dependent on Ca. When there is a decrease in serum Ca concentration there is an increase in the secretion of PTH. PTH restores Ca concentrations in the serum by stimulating osteoclastic breakdown of bone which releases Ca and also P into the circulation. Higher PTH also acts at the kidneys to inhibit renal excretion of Ca but also increase renal excretion of P. The increased production of calcitriol by PTH stimulates intestinal absorption of both Ca and P (Digirolamo et al. 2012). Compared to rats fed normal Ca, serum P concentrations were higher in rats fed the highest (20Ca) and lowest (0.75Ca) Ca diets (data not shown). These results may be explained by effects of Ca on urinary P excretion, intestinal absorption and resorption from bone. Lower circulating PTH decreases urinary P excretion (Pu et al. 2016). Urine P concentrations were decreased in a dose-dependent manner in rats fed higher Ca. Reduction in urinary P may have contributed to the increase in serum P observed in rats fed the highest Ca diet (20Ca). The higher serum P concentrations in rats fed the lowest Ca diet may

be explained by increased intestinal absorption of P and possibly resorption from bone (Pu et al. 2016).

Studies have shown that high Ca intakes increase the excretion of Mg in urine and decrease intestinal Mg absorption (Bertinato et al. 2016). In this study the decline in serum Mg concentrations in rats fed higher Ca (data not shown) is not explained by changes in urinary Mg excretion since urinary Mg excretion was similar among groups. The lower serum Mg is most likely explained by increased competition for intestinal absorption between Ca and Mg (Bertinato et al. 2016).

5.3 Diets higher in Ca did not reduced body weight or fat mass

Ca intake may affect body weight and fat mass, but results from human and animal studies have been inconsistent (Davies et al. 2000; Heaney et al. 2002; Zemel et al. 2004; Zemel et al. 2005; Gunther et al. 2005; Reid et al. 2005; Shapses et al. 2004; Bowen et al. 2005). In an effort to reconcile results from studies, it has been proposed that higher Ca intakes may only have a small effect on reducing body weight and fat mass and larger effects may only occur in specific circumstances such as conditions of energy restriction or when baseline Ca status is low (Villarroel et al. 2014). Furthermore, it has been suggested that dairy products may have a larger effect compared to Ca alone that may be explained by additional anti-obesity compounds in dairy (Zemel 2004).

In this study diets containing between ~40% and 400% of normal Ca, fed *ad libitum*, did not affect body weight or fat mass of the rats. However, rats fed the 0.75Ca diet containing very

low Ca (~16% of normal) had lower body weight and fat mass compared to other groups. It should be noted, however, that this degree of Ca deficiency is rare in the general population.

It has been proposed that higher Ca intakes may promote weight loss by decreasing appetite or decreasing fat absorption and consequently digestible energy (Soares et al. 2014). Overall food consumption was greater in rats fed the high (10Ca and 20Ca) compared to the low (0.75Ca and 2Ca) Ca diets. The differences in food consumption may be explained by the modest differences in energy densities of the diets. This is supported by results demonstrating similar energy intakes among groups (excluding the 0.75Ca group). Overall energy efficiency was lower for rats fed the highest Ca diet (20Ca) compared to rats fed the 2Ca low Ca diet. This may be explained by the lower fat digestibility and presumably lower energy digestibility of the 20Ca diet. Together, the results indicate that the higher Ca diets did not reduce appetite of the rats. These results are in general agreement with a study in diet-induced obese mice that reported higher food consumption and increased body weight and fat depots with higher dietary Ca (Thomas et al. 2012). In that study, only mice fed a diet containing high Ca plus non-fat dry milk showed reduced body weight and adiposity compared to control mice fed a normal Ca diet suggesting other components in the dairy (not the Ca) had anti-obesity properties. Results from the current study also do not support the idea that lower fat and energy digestibility of diets higher in Ca decrease body weight or fat mass in rats.

Some research has suggested that higher Ca intakes decrease lipogenesis and increase lipolysis in adipose tissue leading to reduced body fat (Soares et al. 2014). The proposed mechanism involves a decrease in circulating PTH and 1,25 dihydroxyvitamin D which causes a decrease in intracellular Ca concentrations in fat cells (Soares et al. 2014). In this study, rats fed the 20Ca diet had lower circulating PTH (1,25 dihydroxyvitamin D was not measured in this

study) compared to other groups, but these rats did not have lower total fat mass measured by magnetic resonance imaging. The weights of 4 distinct fat depots (i.e., epididymal, inguinal, mesenteric and perirenal + retroperitoneal) also were not lower compared to other groups. Thus, if the differences in dietary Ca affected lipogenesis or lipolysis in adipose tissue, the effects were small and physiologically irrelevant.

5.4 Dietary Ca affected serum and liver lipids

Changes in blood lipids are well-established risk factors for CVD. High concentrations of serum LDL-C, TC, TG and low concentrations of HDL-C increase the risk of CVD (Jocelyne et al. 2017). Lowering specifically LDL-C has been shown to significantly reduce the risk of CHD (Jocelyne et al. 2017). Emerging evidence suggests that dietary Ca may play an important role in improving serum lipid profile suggesting that Ca intakes may influence the risk of CVD. Some human and animal studies have shown improved serum lipid-lipoprotein profile with higher Ca intakes (Groot et al. 1980; Denke et al. 1993; Shahkhalili et al. 2001; Ditscheid et al. 2005) but not all studies have confirmed these findings (Reid et al. 2010; Palacios et al. 2011; Bostick et al. 2000). In this study rats fed higher than normal Ca (10Ca and 20Ca diets) had a serum lipid profile predictive of a lower risk for CVD. Serum TC and LDL-C were lower as well as TC:HDL-C and LDL-C:HDL-C ratios.

Abnormal glucose metabolism and insulin resistance is associated with elevated serum concentrations of triglyceride-rich lipoproteins and reduced HDL-C. Studies have shown an inverse relationship between Ca intake and plasma glucose and insulin concentrations in rats (Marotte et al. 2014). In this study plasma glucose and insulin concentrations did not differ

among rats fed between ~40% and 400% of normal Ca suggesting little effect of dietary Ca at these doses on glucose metabolism and insulin sensitivity. It is therefore unlikely that changes in glucose metabolism or insulin sensitivity accounted for the observed differences in serum lipids of the rats. Rats fed very low Ca (0.75Ca) did have lower serum insulin concentrations compared to the other groups. This is likely explained by impaired secretion of insulin from pancreatic β-cells as previously reported with moderate to severe Ca deficiency (Draznin 1988).

Dietary Ca induced changes in mRNA expression of lipogenic genes in the liver of the rats. Rats fed higher Ca had higher expression of *CPT2* suggesting increased mitochondrial FA oxidation. Rats fed the low Ca diets (0.75Ca and 2Ca) had lower expression of *FASN* compare to rats fed the normal (5Ca) or high (10Ca and 20Ca) Ca diets suggesting decreased endogenous FA synthesis when fed a Ca-deficient diet. Expression of the LDLR that functions in hepatic cholesterol uptake from the circulation and genes involved in endogenous cholesterol synthesis (*HMGCR* and *HMGCS1*) and cholesterol esterification (*ACAT2*) were higher in rats fed higher Ca. These changes in gene expression are consistent with a response by the liver to a direct or indirect depletion of cholesterol in rats fed higher Ca. Rats fed the high Ca diets had lower TC concentrations in the liver.

Compared to rats fed normal Ca (5Ca), liver weight was lower in rats fed the high Ca diets. This is consistent with other studies that have reported an inverse relationship between Ca intake and liver weight (Marotte et al. 2014). Given that total lipid concentrations in the liver did not differ among the groups, the differences in liver weight cannot be fully explained by differences in total lipid accumulation. It is possible the changes in dietary Ca may affect global gene expression and proliferation of the liver (Soares et al. 2014; Marotte et al. 2014).

5.5 Higher dietary Ca increased fecal excretion of NS and BA

The interaction of Ca with lipids and BA in the GI tract may account for the improved serum lipid profile of rats fed diets higher in Ca. Ca can bind FA in the GI tract forming insoluble Ca/fatty acid soaps reducing the absorption of FA. Higher dietary Ca may also reduce the intestinal absorption of NS such as cholesterol and decrease the reabsorption of BA. Decreased intestinal absorption of cholesterol and other NS (i.e., cholesterol metabolites) as well as decreased reabsorption of BA could result in greater loss of cholesterol in rats fed higher Ca. The results showed that compared to rats fed normal Ca (5Ca), fecal excretion of cholesterol, total NS (sum of cholesterol, coprostanol and cholestanol) and total BA was higher, while apparent cholesterol retained was lower in rats fed the highest Ca diet (20Ca). It should be mentioned that these parameters did not differ between rats fed the normal (5Ca) or 10Ca diets. These results suggest that reduced absorption of NS and decreased reabsorption of BA may have contributed to the lower serum TC and LDL-C observed in rats fed the highest Ca diet (20Ca) by increasing hepatic cholesterol demand (for synthesis of BA and other cholesterol metabolites) and consequently increasing uptake of cholesterol from the circulation through the upregulation of the LDLR. However, the lack of differences in fecal excretion of NS and BA between rats fed the 5Ca or 10Ca diets suggest an alternative mechanism may account for the differences in serum lipids between these groups.

5.6 Higher dietary Ca decreased FA digestibility

Replacing SFA and TFA in the diet with MUFA and PUFA has a beneficial effect on serum lipid profile and thus a protective effect on CVD risk (Brouwer 2016). Ca can bind and precipitate FA in the GI tract reducing their absorption. Studies in humans and animals have shown increased fecal total lipid and FA excretion with higher intakes of Ca (Denke et al. 1993; Shahkhalili et al. 2001; Jacobsen et al. 2005; Bendsen et al. 2008; Govers & Meer 1993). Some results suggest that SFA are more susceptible to precipitation by Ca, but not all results have been consistent. A study in humans reported that MUFA were more affected by Ca than SFA (Bendsen et al. 2008).

If Ca preferentially reduces the absorption of SFA or TFA, higher intakes of Ca could be expected to have a beneficial effect on serum lipid profile. In this study the use of a ruminant source of fat (i.e., anhydrous milk-fat) in the basal diet allowed for the investigation of the effects of Ca on all 4 major classes of FA including TFA. To date, to the best of my knowledge, there is no information on the effects of Ca on TFA digestibility.

TFA can be generally classified as natural (derived from ruminant sources) or industrial which are formed during hydrogenation processes or inadvertently during oil refining. Although the proportions of natural versus industrial TFA vary depending on the food source, they are not exclusively found in one food versus another. It is well-established that industrial TFA have a negative effect on serum lipids and pose a health risk (Brouwer 2016). Health Canada has recently banned the use of partially hydrogenated oils in foods (Health Canada, 2017). Whether natural sources of TFA found in milk and other dairy products also pose a health risk is currently a topic of debate (Stender 2015).

Rats fed diets with higher Ca showed decreases in the apparent digestibility of all 4 major classes of FA. The largest effects were seen for TFA followed by SFA. The differences in TFA digestibility were substantial. Rats fed low, normal or high Ca had digestibilities of 98%, 78% and 42–46%, respectively. It is possible that the lower TFA digestibility in rats fed the high Ca diets contributed to the lower serum TC and LDL-C observed in these rats.

Studies have investigated the possible mechanisms accounting for the adverse effects of SFA and TFA on blood lipids. One of the proposed mechanisms is that SFA and TFA increase serum LDL-C by decreasing the activity of the LDLR in hepatocytes. It has been shown that dietary SFA and TFA negatively correlate with LDLR activity (Fernandez & West 2005). This may be explained by a decrease in the formation of CE. SFA have been shown to suppress ACAT activity (the rate-limiting enzyme in cholesterol esterification). This may result in a larger proportion of cholesterol remaining in the regulatory pool causing a down-regulation of the LDLR. In this study liver ACAT2 mRNA expression increased in rats fed higher Ca; however, FC in the liver did not differ among diet groups and TC was decreased in rats fed higher Ca. SFA and TFA may also affect the production of apoB-100 containing lipoproteins. A study examined the effect of consuming low SFA and high PUFA (Ooi et al. 2015). High PUFA consumption decreased LDL-C by rising apoB-100 lipoprotein catabolism. Another study examined the effect of TFA on lipoprotein metabolism in post-menopausal women (Ooi et al. 2015). The results showed an increase in TC and LDL-C and a decrease HDL-C. This effect was associated with a defect in ApoB-100 catabolism (Ooi et al. 2015). TFA may also increase circulating LDL-C by increasing cholesterol synthesis. When TFA intake was compared with intake of palmitic acid (a SFA), an increase in cholesterol synthesis and plasma LDL-C concentrations were observed (Sundram et al. 2003). This suggests that increased cholesterol

synthesis contributed to the increase in LDL-C with TFA intake. Given the large, dose-dependent, inverse relationship between Ca intake and TFA and SFA digestibility, dietary Ca may improve serum lipid profile by decreasing absorption of TFA and SFA. The effect of dietary Ca may be greater in persons consuming a diet high in TFA or SFA.

Digestibility of both natural and industrial TFA were affected by Ca with the largest effect seen for vaccenic acid (18:1 11t), the primary natural TFA. The negative values for the digestibility of vaccenic acid in rats fed the high Ca diets indicate that more of the FA was excreted in the feces than consumed from the diet. This is likely explained by the conversion of other FA such as CLA to vaccenic acid in the intestine of the rats (Devillard et al. 2007). The effect of the lower digestibility of vaccenic acid on health is not unclear. Some studies have suggested that vaccenic acid does not exert the same adverse health effects on cholesterol synthesis compared to industrially produced TFA, although the topic is still controversial (Krogager et al. 2015). Also, some studies have suggested anti-carcinogenic properties of vaccenic acid (Lim et al. 2014). Another study has suggested a detrimental effect of vaccenic acid on bone (Hamazaki et al. 2016).

The higher Ca diets reduced the digestibility of SFA. In general, the largest effects were seen for longer chain SFA that may be explained by the longer transit time in the intestine allowing more time for interaction with Ca (Bribiesca et al. 2017). However, a number of other factors including degree of saturation, chain length and position on the glycerol backbone may also affect the interaction of fatty acids with Ca.

Digestibility of MUFA and PUFA were also lower in rats fed higher Ca, but the magnitude of the effect was much smaller. There are 2 types of essential FA, omega-3 FA and omega-6 FA. A higher amount of omega-6 FA in the diet or higher omega-6:omega-3 ratio has

been associated with health conditions including CVD, inflammation, cancers, and autoimmune diseases (Simopoulos 2002). A higher amount of omega-3 FA in the diet or lower omega-6:omega-3 ratio has been associated with beneficial health effects (Simopoulos 2002). Interestingly, the digestibility of omega-3 FA was more affected by Ca than omega-6 FA. Thus, a higher Ca diet may increase the dietary omega-6:omega-3 ratio.

5.7 Limitations and strengths of the study

The main limitation of this study is that the effects of dietary Ca on pathological endpoints of CVD were not examined. Although the observed effects on blood lipids would suggest a reduced risk for CVD in rats fed the high Ca diets I cannot say for certain that the higher Ca intakes were protective of CVD in these rats. Further research on the effects of higher intakes of Ca, especially from high-dose Ca supplements, on CVD endpoints is warranted. Results for FA digestibility and excretion of NS and BA were determined with fecal samples collected over a 1-week period of a 10-week study. However, differences in fecal bulk among diet groups measured during week 3 and week 8 were comparable suggesting that the effect of dietary Ca on FA digestibility and excretion of NS and BA was likely consistent throughout the study. Also, only male rats were used in this study and therefore it is not possible to extrapolate results to females. Male rats were chosen because female rats, when fed high Ca diets, develop kidney calcification and therefore are not a good animal model for this research (Cockell & Belonje 2004). Another limitation of this study is that only mRNA expression of lipogenic genes in the liver was measured. Measurement of protein content would have provided a better indication of changes in enzymatic activity.

Rat is commonly used as an animal model in lipoprotein research. Experimental data collected from rats are frequently extrapolated to humans. However, there are differences in terms of lipoprotein type, composition and concentration between rats and humans. Lipoproteins can be classified into VLDL, LDL, and HDL. In rats there is an additional major type of lipoprotein in serum called HDL_1 (Innerarity et al. 1980). Another difference between rat and human is apoprotien E which exists as a major component in rat HDL, while apoprotien E is present in smaller amounts in human HDL (Innerarity et al. 1980). Also, the distribution of lipoproteins is different between rats and humans. LDL is the major type of lipoprotein that transports cholesterol in the circulation in humans, while HDL is the predominant type in rat (Innerarity et al. 1980).

A main strength of the study is that FA digestibility (including TFA), fecal excretion of NS and BA and lipid metabolism in the liver were investigated in the same study across multiple doses of Ca. This experimental approach provides a better indication of the specific doses of Ca and underlying mechanisms driving changes in blood lipids.

5.8 Conclusions

Ca intakes for a large segment of the North American population fall short of dietary recommendations (US; CCHS 2.2, 2004); while upper percentiles of intakes for some subpopulations (e.g., older women) exceed the upper limit from use of high-dose Ca supplements (Martineau et al. 2014). Given the wide range of Ca intakes in the population, it is important to understand the effects on CVD risk. This study has shown that differences in dietary Ca alone (i.e., without changes in other components in dairy foods) has little effect on body weight or fat

mass unless Ca intakes are very low (a degree of Ca deficiency that is rare in the general population). Diets containing above normal amounts of Ca induced changes in blood lipids predictive of a lower risk for CVD. The mechanisms accounting for the changes in blood lipids may depend on the specific Ca dose (Figure 15). At the highest Ca dose (20Ca, ~400% of normal), decreased absorption of NS (such as cholesterol) and increased excretion of BA may have accounted for the lower serum TC and LDL-C. Decreased digestibility of TFA and SFA may also have contributed to the improved lipid profile at the highest Ca dose. At a lower Ca dose above normal (10Ca, ~200% of normal), decreased digestibility of TFA and SFA may have accounted for the improved serum lipid profile given the lack of differences in fecal NS and BA excretion and cholesterol balance compared to rats fed normal Ca (5Ca). A major finding reported in this study is the sizeable decrease in TFA digestibility with higher dietary Ca. It will be important to determine in future research if the Ca in dairy products attenuates the adverse effects of TFA in these products. This is important since natural (ruminant) TFA may have the same adverse effects on blood lipids as industrial TFA (Stender 2015), but are more difficult to remove from the food supply.

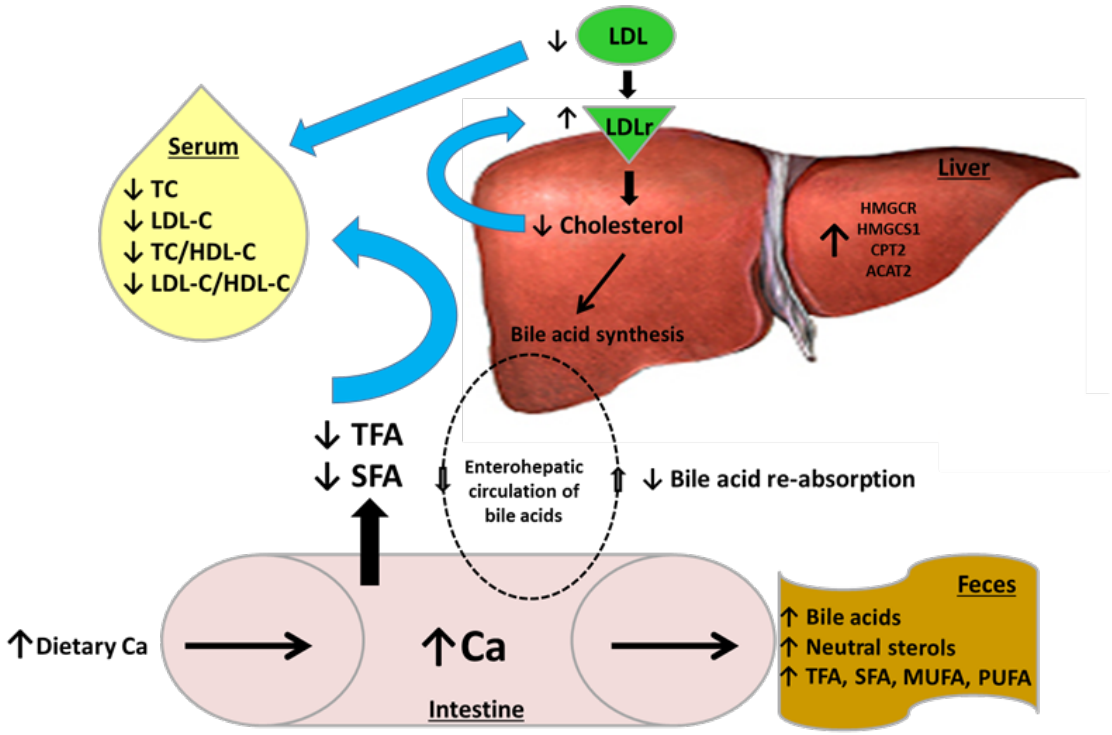


Figure 15. Proposed mechanisms for the improved serum lipid profile of rats fed higher Ca. Higher Ca in the diet increases Ca concentration in the intestine. This leads to a decrease in the absorption of BA, NS and FA. This results in less BA circulating back to the liver by enterohepatic circulation. Therefore, more BA need to be synthesised using hepatic cholesterol resulting in hepatic cholesterol depletion. The increase in NS excretion may also deplete hepatic cholesterol. The lower hepatic cholesterol leads to an increase in expression of the LDLR which increases the removal of LDL-C from the circulation. The lower digestibility of TFA and SFA with higher Ca intakes may also improve blood lipid profile since higher consumption of these fatty acids (at the expense of MUFA or PUFA) has been shown to increase TC and LDL-C. Ca, calcium; LDL, low density lipoprotein; LDL-C, low density lipoprotein cholesterol; LDLr, low density lipoprotein receptor; TFA, trans fatty acid, SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

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