THE INFLUENCE OF DIETARY FAT AND INTESTINAL pH ON CALCIUM BIOACCESSIBILITY: AN *IN VITRO* STUDY

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ABSTRACT OF THE THESIS

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Human studies measuring true fractional calcium absorption have shown that dietary fat is a significant predictor of calcium absorption. In murine models, dietary fat also increases calcium absorption, but whether there is a differential effect with the type of fatty acid (FA) on intestinal calcium absorption is less clear. Compared to monounsaturated FAs (MUFAs), saturated FAs (SFAs) decrease lipid membrane fluidity and have a greater intestinal transit time, potentially prolonging their interaction with calcium in the gut. In addition, SFAs bind to calcium forming insoluble soaps, increasing fecal fat excretion, and reducing calcium availability for absorption. In addition, luminal factors such as a higher pH in the GI tract could influence calcium absorption such as with certain medications or achlorhydria. The TNO gastrointestinal model (TIM-1) replicates the physiological activities occurring in the lumen of the stomach, duodenum, jejunum, and ileum and is used to study biological events preceding nutrient absorption (i.e., bioaccessibility). In this study, we examined two high fat formulas (SFA or MUFA enriched) compared to low fat and controlled for calcium (500 mg) and other

micronutrients during a 5-hr experiment. Calcium bioaccessibility (CaB) was greater for the high compared to low fat test meal in the jejunum ($p = 0.001$). In addition, CaB with the SFA alone was higher than either LFD or MUFA ($p \leq 0.01$). However, there was no interaction between diet and CaB in the ileum or ileal efflux. During high gastrointestinal pH, CaB was similar between diets in the jejunum and ileum, and there was a trend for increasing non-bioaccessible calcium over time ($p = 0.058$). Furthermore, CaB was 90% ($p = 0.003$), 91% ($p = 0.036$), and 94% ($p = 0.001$) lower in the jejunum, ileum, and ileal efflux, respectively at high pH compared to normal gastrointestinal conditions. These findings suggest a dramatically lower CaB under high pH conditions, having implications for a wide range of patients with gastrointestinal disorders. In addition, the higher Ca absorption associated with a high fat diet found previously, may be partially explained by an increased CaB.

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CHAPTER 1: INTRODUCTION

Calcium metabolism occurs in three critical organs: small intestine, kidney and bone [67]. Calcium homeostasis in the body is controlled by 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), also known as calcitriol, parathyroid hormone (PTH), and calcitonin [67]. When plasma calcium is low, a calcium-sensor protein in the parathyroid gland initiates the secretion of PTH. In turn, PTH directly stimulates bone resorption and the release of calcium from the bones into the circulation and optimizes calcium reabsorption from the kidney with minimal urinary loss [6, 39]. In addition, PTH promotes 1-α-hydroxylation of 25-hydroxyvitamin D (25(OH)D), to $1,25(OH)_2D$ and increases its synthesis [68]. Consequently, $1,25(OH)_2D$ acts in the intestine and stimulates local calcium absorption. In the kidney and bone, $1,25(OH)_2D$ acts in a similar manner to PTH and enhances calcium reabsorption from the glomerular filtrate while promoting bone resorption and calcium release into the blood, respectively [40]. Calcitonin, a hormone synthesized in the thyroid gland also regulates calcium levels, but opposes the effects of PTH [6, 43]. It decreases osteoclast activity and therefore bone resorption and reduces the release of calcium into the blood [68].

1.1 Calcium absorption

The process of calcium absorption takes place throughout the intestinal tract and involves dietary calcium transport across the intestinal lining. The overall calcium that is absorbed by the small intestine is contingent on dietary calcium intake. Calcium transport occurs via two pathways, transcellular and paracellular. [8]. Saturable, transcellular or active

transport requires metabolic energy in the form of ATP, occurs predominantly in the proximal duodenum, and is vitamin D dependent [53]. Active transport is the major route when calcium intake is low and is downregulated when total calcium intake is increased [54]. This route is facilitated by $1,25(OH)_2D$, TRPV6/CaT1, a vitamin D dependent membrane channel protein, calbindin $D_{9k,}$ a calcium binding protein, and basolateral Ca^{2+} ATPase [8, 67]. On the other hand, non-saturable, paracellular or passive transport does not require additional energy and is independent of age, nutritional and/or hormonal status [8]. This pathway tends to predominate when calcium intake is high.

Moreover, calcium absorption differs throughout the small intestine with the majority of calcium being absorbed in distal jejunum and ileum [8]. Furthermore, the rate of calcium absorption differs with the calcium formulation [46]. In the liquid form (eg. milk), calcium absorption decreases after 2 hours compared to the solid form (eg. cheese) where there is a slower decrease in the rate of absorption at 6 hours [46]. Also, the amounts of calcium absorption in each segment are lower for the liquid form compared to the solid form. Although, the distal jejunum and ileum are responsible for the majority of calcium absorption, studies have shown that 10% of total calcium absorption occurs in the large intestine via both active and passive transport [8]. A study conducted in patients with inactive Crohn's disease found significantly decreased absorption in patients who underwent ileostomy (10%) compared to those with a preserved colon (14%) [30] and subsequent studies confirmed these findings [31].

1.2 Physiological and pathological conditions impacting calcium metabolism

As discussed previously, calcium homeostasis is under tight hormonal regulation. Physiological changes in the hormonal milieu (aging, menopause), pathological hormonal abnormalities (obesity, diseases) as well as dietary factors can potentially alter calcium metabolism. Current literature on factors influencing calcium absorption will be reviewed in this section.

1.2.1 Age and menopause

Aging has been associated with a decrease in calcium absorption. A study conducted in 115 women and 75 men aged 20-95 years showed a decrease in calcium absorption at age 60 and significant calcium malabsorption at age 80 [10]. The significant decrease in calcium absorption in this sample was attributed to the increased prevalence of vitamin D deficiency in the elderly [10]. The exact mechanism for this finding is unclear. The authors speculated that the decreasing levels of serum $1,25(OH)_2D$ and intestinal vitamin D receptor (VDR) may be considered responsible factors for decreased calcium absorption in the elderly [10]. Moreover, in a more recent study by Bullamore, when young women (25-45 yrs) were compared to older women (65-83 yrs), a similar decrease in calcium absorption was found with older age [37]. Similar to the Bullamore study the decreased plasma levels of $1,25(OH)_2D$ explained the decrease in calcium absorption, however, the intestinal VDR was not associated with calcium absorption [37].

In addition to aging, the effect of menopausal status on calcium absorption has been addressed in several studies. A decrease in estrogen with menopause could negatively

impact calcium transport in the GI tract and could therefore decrease calcium absorption. Nordin and colleagues assessed 262 healthy postmenopausal women (40-87 yrs) and reported that the 30% reduction in calcium absorption found in women older than 75 years can no longer be explained by low serum $1,25(OH)_2D$ with aging, but may be due to a decline in calcium transport related to menopausal status [51].

1.2.2 Obesity

Obesity is associated with altered levels of the essential hormones necessary for calcium homeostasis. These hormones include decreased 25-hydroxy vitamin D and $1,25(OH)_2D$ and increased PTH, estradiol, and sex steroids [3, 44, 55]. Recent studies are reporting that dietary fat intake is associated with increased calcium absorption [57, 63]. A study in women (24-75 years) using dual isotopes to measure true fractional calcium absorption (TFCA) examined dietary and hormonal factors that influence TFCA. [63]. The study reported higher TFCA in obese individuals and concluded that dietary fat, estradiol, and $1,25(OH)_2D$ are positive determinants of TFCA in women [63]. Similarly, another study evaluating factors associated with TFCA and net calcium absorption (NCA) in post-menopausal women suggested that the consumption of fat is associated with increased calcium absorption [57] Therefore, because of excess fat consumption, obese patients may have increased calcium absorption.

These findings are further supported by observational data presented by Wolf et al. Wolf and colleagues also examined potential factors that could affect calcium absorption in pre- and peri-menopausal women [75]. This study was different in that they investigated

effects of vitamin D receptor gene polymorphisms, dietary fiber, and alcohol consumption, in addition to body mass index (BMI), dietary fat intake, $1,25(OH)_2D$, and PTH. They found that TFCA is positively associated with BMI and dietary fat intake, as well as $1,25(OH)_2D$, and PTH [75]. At this time, a succinct mechanism to explain this increase in calcium absorption is lacking, but these results do indicate that dietary fat is a significant predictor of TFCA.

1.3 Fatty acids (FAs) and their interaction with dietary calcium

Recent studies show that dietary fat is a positive predictor of calcium absorption; however, the mechanism by which this occurs is still uncertain. The interaction between calcium and FAs in the gut involves the increase of fecal fat excretion due to the formation of insoluble calcium soaps with FAs; thereby, decreasing the amount of calcium that is available for absorption [4]. Bendsen et al examined the effects of two dairy diets (low vs. high calcium) in a randomized controlled study in humans on fecal fat excretion and concluded that there was significantly greater fecal fat excretion with the high calcium diet compared to the low calcium diet [4]. The study showed that there was greater MUFA content compared to SFA at higher levels of calcium. This was a surprising observation because a previous study by Denke et al reported that increased calcium intake is associated with greater SFA excretion which could be explained by the lower solubility of calcium SFA soaps [15]. However, Denke et al did not look at the influence of various dietary fats on calcium absorption and the chemical properties of saturated vs. unsaturated FAs which could influence the degree of soap formation

differently [15]. Therefore, the amount of calcium that may be available for absorption by the small intestine could vary with the type of fat.

Saturated FAs are absorbed slower than unsaturated FAs prolonging their contact with biliary micelles and gut secretions [52]. Because saturated FAs have a longer intestinal transit time, it is possible that their interaction with calcium is also prolonged, promoting soap formation and decreasing the calcium that could be available for intestinal absorption. As reported by Denke et al, the increased fecal fat excretion as observed with a high SFA diet in his study may be due to the fact that SFAs require a longer duration for optimal absorption in the small intestine in comparison to unsaturated FAs [15, 52]. The greater intestinal transit time makes SFAs more susceptible to disruption in the gut [52] through the formation of insoluble calcium+FA soaps and changes in membrane lipid composition. For example, saturated FAs have been associated with decreased lipid fluidity compared to unsaturated FAs [7, 12]. The formation of these soaps may render both calcium and SFA unavailable for intestinal absorption. These findings; therefore, signify that soap formation can interfere with calcium absorption in the gut.

The formation of intestinal calcium soaps which may be associated with a reduction in calcium absorption is further influenced by FA chain length and degree of unsaturation. Gac et al assessed the amount of calcium that would be available for absorption from soaps of different dietary fat origin [24]. In this study, soaps were administered directly into the duodenum and the following was concluded (1) there was an inverse relationship between calcium absorption and chain length; a decrease in absorption was observed with

longer chain FAs, (2) increasing the degree of unsaturation improved absorption [24]. Moreover, different fats have varied effects on calcium absorption; stearic acid caused a decrease in calcium absorption along with increased soap formation while triglycerides (tristearate, trioleate, and tridecanoate) had minimal effect on both absorption and soap formation [24]. Although, an exact mechanism to elucidate why certain FAs influence the intestinal calcium absorption differently was not provided; it was concluded that fecal fat excretion was a direct reflection of the soap formation [24] and this could explain the decrease in intestinal calcium absorption.

Furthermore, the extent to which types of fat form insoluble soaps with calcium and are excreted varies upon the positional distribution of the FA on the dietary triacylglycerol (TAG) [4]. The majority of fat that is consumed from the human diet is in TAG form and contains three FAs connected to a glycerol. A major component of FA digestion is the enzymatic hydrolysis of TAG which takes place in the duodenum [74]. Enzymatic hydrolysis requires the action of lipases that target the FAs on the sn-1 and sn-3 positions to yield free FAs and sn-2 monoacylglycerols [74]. The FAs which are located on the terminal ends of a TAG (sn-1 and sn-3 positions) bind readily to calcium because they are hydrolyzed from the triacylglycerol molecule via lipase making these FAs available for absorption [48]. On the other hand, FAs in sn-2 position are not available as free FAs in the intestine because they remain in the form of a monoacylglycerol until absorption [48] potentially inhibiting soap formation. FA position is an important determinant of soap formation in the intestine. The source of fat used in Bendsen's study was butter which is rich in SFA, dominates the sn-2 position, and is not available as free FAs in the

intestinal lumen [4, 48]. Bendsen et al reported that there was greater fecal fat excretion with the MUFA as compared to the SFA diet [3]. Because butter is dominated by SFA in the sn-2 position, free FAs are less available to interact with calcium potentially resulting in decreased soap formation and thereby decreased fecal fat excretion. The FA position (SFA in sn-2) could therefore explain why there was a decreased effect of calcium on fecal fat excretion for the SFA diet.

In addition to the position of the FA, chain length also plays an important role in determining the fate of the FA in the intestine; in turn, influencing the degree of calcium absorption in the gut. A review reports that SFAs on the sn-1 and sn-3 compared to those on the sn-2 position display different biological consequences in the intestinal lumen due to their lower absorptive capacities [14]. For example, free long chain FAs such as palmitic (C-16) and stearic (C-18) in the sn-3 position on TAG have a propensity to form calcium soaps and have melting points higher than body temperature giving rise to their lower absorption rates [14, 35]. In contrast, short chain FAs tend to be absorbed rapidly in the intestine due to their high capacity to solubilize in the intestine [14]. More importantly, FAs in the sn1/sn3 versus sn2 exhibit different absorption patterns in the intestine. During digestion, the majority of the FAs in the sn1/sn3 positions of TAG are hydrolyzed while only 22% of those in the sn2 undergo complete hydrolysis [35, 48]. FAs dominating the sn-1 and sn-3 positions therefore, may be more vulnerable to alterations by gut secretions. These absorption patterns may be responsible for intestinal gut disturbances that could change the content and composition of fecal fat excretion as well as the formation of insoluble soaps.

The interaction between calcium and FA is a complex one because different food matrices seem to exert different physiological effects in the gut. Milk and cheese similar in fat and calcium contents, for example, have different effects on intestinal calcium absorption because of their chemical and physical characteristics [66]. It is likely however, that these interactions may be due to the components and nutrients present in the dairy source that are modulating the changes taking place in the gut. A randomized crossover study using three types of dairy sources, control (non-dairy), semi-skimmed milk, and semi-hard cow cheese diets containing 500 in the control and 1700 mg Ca/d in both dairy groups looked at how SFA in each of these diets affects lipid levels [66]. The study reports that both dairy diets (milk and cheese) mitigate the SFA induced increase in total and LDL cholesterol while increasing fecal fat excretion compared to the control diet [66]. Again, this was attributed to calcium's ability to form calcium FA soaps. Although, Soerensen et al's study demonstrated no differences in source of dairy on calcium induced fecal fat excretion, other studies suggest that calcium derived from different sources may affect blood lipids and fecal fat excretion differently [5,34,). Specifically, fat in milk is predominantly present as small globules that are bound by a membrane while fat in cheese is encompassed by a protein structure, casein [28]. Calcium in cheese is bound to casein prolonging the interaction between fat and calcium when cheese is consumed while calcium in milk is in a liquid state [68]. As previously discussed, whether the dairy source of calcium is in the liquid or solid form also influences these dynamics. Therefore, gastric emptying rate is faster for the liquid phase compared to solid phase dairy sources; thus, calcium may reach the duodenum before fat, hindering optimal interaction between calcium and fat as well as calcium-fatty acid soap

formation [66]. Since the study performed by Soerensen was conducted in young healthy individuals, there were no significant differences between the milk and cheese diets on lipid profile and fecal fat excretion. However, in a population prone to atherogenic dyslipidemia, results may have differed between diets. To date, literature has greatly focused on the effect of SFA on calcium, but the relationship between MUFA and PUFA and calcium has been less studied.

1.4 Fatty acid absorption and achlorhydria

Achlorhydria is reported to be a condition defined by the absence of HCl in gastric secretions and a pH of $\geq$ 7 [76]. However, others have reported a gastric pH of greater than 6.81 in females and 5.09 in men [40]. This condition negatively affects calcium absorption in the small intestine. Adequate calcium absorption requires the presence of gastric acid which causes dissolution of calcium into a calcium ion (Ca) in the stomach [64]. Without sufficient gastric acid secretion, calcium salts cannot be dissolved or ionized, leading to poor calcium absorption in the small intestine [64]. In healthy individuals, calcium salts (calcium carbonate) which are poorly soluble in the gut are converted to calcium chloride and dissociated to form $Ca^{2+.}$ Calcium ions are water soluble and therefore, easily solubilized in the intestine [64]. A decrease in acid production is common in the elderly; especially those taking antiulcer medications and proton pump inhibitors (PPIs). Because conditions like achlorhydria have been associated with calcium malabsorption, several studies have been conducted to examine whether calcium absorption varies with different calcium sources during high intestinal pH. It is reported that increased gastric pH has a pronounced effect only when poorly soluble

calcium is ingested after an overnight fast, while soluble calcium sources from milk and calcium citrate are still absorbed sufficiently [76]. Other minerals like iron also show decreased absorption in achlorhydric patients and contribute to iron deficiency anemia [5].

Furthermore, a high intestinal pH as a result of achlorhydria may influence the absorption of saturated and unsaturated FAs differently. Saturated FA chains are closely packed together and have stronger binding forces compared to unsaturated FAs, resulting in greater pKa for saturated vs. unsaturated FAs [38]. Normal gastrointestinal pH conditions favor absorption of acidic compounds (low pKa) [38]. In contrast, during achlorhydric conditions, high pH induces translocation of basic compounds (high pKa) favoring absorption while drastically decreasing absorption of acidic compounds [78]. Because absorption of saturated FAs (high pKa) is not favored under normal pH conditions, it can be concluded that healthy subjects display greater unsaturated FA absorption compared to saturated FA. Patients with achlorhydria, on the other hand, may exhibit diminished absorption of unsaturated FAs as compared to saturated FAs. The difference in absorption patterns for these dietary fats remains unclear and warrants more research; however fat absorption may be compromised in achlorhdric patients due to a decreased absorptive surface and reduced secretion of digestive enzymes including lipase, trypsin, chymotrypsin, and amylase [63].

1.5 TNO Gastrointestinal model (TIM-1)

In vitro studies provide an ideal platform to study nutrient interactions in foods affecting calcium absorption giving researchers insight on the various nutritional disorders that could arise from the impaired relationships between calcium and other nutrients.

Firstly, it is important to comprehend the bioavailability, bioaccessibility, and bioactivity when using these *in vitro* methods and instruments. Bioavailability refers to the fraction of the ingested nutrient that goes into systemic circulation to exert its biological effects on the designated tissue [56]. Bioaccessibility, on the other hand, represents the amount available for intestinal absorption *in vivo* [56]. In other words, bioavailability cannot be assessed solely using *in vitro* methods because it entails a physiological basis and thus, physiological factors including age, pregnancy, lactation, genotype, and pathological conditions including nutrient deficiencies and disease states [18]. Bioactivity involves the transport of the compound, interaction with biomolecules, metabolism, and finally the physiological response [20]. Figure 1 shows the relationship between bioavailability, bioaccessibility, and bioactivity that encompass the processes of transforming food into components that are digested and absorbed in the intestinal lumen, and exert their biological effects to designated tissue [20]. These *in vitro* methods are enabling researchers to draw information about nutrient-nutrient and nutrient-food interactions, the influence of luminal factors like pH and enzymes, food processing and preparation techniques, components of the food matrix on bioaccessibility [18].

One such instrument is the TNO gastrointestinal model of nutrient bioaccessibility (TIM-1), a computer-controlled system mimicking the GI tract. Human digestion is a complex

process involving: food and water intake, release of digestive enzymes and secretions, mixing facilitated by peristaltic contractions, and finally transit through the stomach and small intestine [17]. These physiological events are replicated by the TIM-1. In addition, pH in the GI tract, electrolyte concentrations, presence of artificial saliva, gastric acids, and pancreatic juices, bile salts, and body temperature are also reproduced [17]. TIM-1 consists of several compartments including the stomach, duodenum, jejunum, and ileum that are connected by valves controlling gastrointestinal transit time [46]. Such *in vitro* methods are highly reliable. The controlled experimental environment eliminates confounding variables that may exist with *in vivo* models, is faster, and less expensive [62]. However, *in vitro* studies cannot replace those *in vivo* because of the absence of gut transporters and hormones that may further influence nutrient absorption. Hence, instruments like TIM-1 can be used for validation results or screening [18].

The TIM system is prominent in pharmaceutical research to determine how drugs interact with food in the GI tract [17]. Originally designed to study food digestion; recently it is been applied to study the bioaccessibility of poorly soluble drugs from different formulations during the fasted or fed states [72]. In our study we replicated the pathophysiological condition of achlorhydria, characterized by the absence of HCl in gastric secretions and an intragastric pH of greater than 6.81 in women and 5.09 in men [39]. Under these conditions, we examined calcium bioaccessibility with different dietary fats. This system is highly efficient as it allows the collection of samples from each compartment at any time during the course of digestion [17]. Thus, this enables the possibility of studying the interactions between intestinal segment and the compound at

each time point. Moreover, TIM-1 can be adapted to emulate gastrointestinal conditions in infants, young adults, and the elderly [29]. However, nutrient absorption can be confounded by many physiological factors with an *in vivo* model. It is well known that calcium absorption in humans is tightly regulated by PTH, vitamin D, proteins, and transporters; nonetheless interactions between dietary factors that may impact calcium fate preceding absorption are less clear. In an *in vitro* model, the content of nutrient or compound that diffuses across the semi-permeable membrane in the intestinal region is representative of the amount that is bioaccessible [17]. With instruments like the TIM, nutrient release, solubility, and availability for absorption can therefore, be examined and translated to understand its effects on bioavailability [69].

1.6 Other dietary factors influencing calcium absorption

Previously, TIM-1 has been used to address the relationship between calcium bioavailability and factors such as phytate and casein phosphopeptides. Phytate, a major component of plants, inhibits calcium absorption because of its ability to form insoluble soaps with calcium; hence, interfering with optimal calcium bioavailability [2]. A study using an *in vitro* simulated gastrointestinal model evaluated the inhibitory effects of oxalate, phytate, tannin, and dietary fibers from cooked versus raw green leafy vegetables (GLV) in a typical Indian diet on calcium bioavailability [2]. Twenty different GLVs that were high in calcium were examined and researchers concluded that the bioavailability of calcium was greatly limited because of high contents of oxalate, phytate, tannin, and dietary fibers. To be specific, phytate which has previously been shown to form insoluble complexes with calcium did not demonstrate as great an inhibitory effect; oxalate from

the GLVs was the greatest inhibitor followed by dietary fiber on calcium absorption [2]. On the other hand, casein phosphopeptides (CPPs) which forms as a result of hydrolysis of casein, a protein found in cow's milk has varied effects on calcium absorption [49]. It was assumed that CPPs promote calcium absorption because of the presence of phosphoserine residue clusters that bind to calcium efficiently; thus hindering the formation of insoluble calcium phosphates [21]. However, Narva et al reports that no significant differences were observed in acute calcium metabolism when postmenopausal women were given either control milk or CPP-enriched milk suggesting that CPP has no role in increasing calcium absorption [49]. In contrast, *in vitro* methods report that CPPs cause an increased influx of extracellular calcium promoting absorption in HT-29 tumor cells [21]. There is considerable evidence on phytate and CPP interaction with calcium, but research on the association of calcium with different FAs is limited.

Moreover, it is important to note that food digests from the TIM-1 can be used to incubate with human intestinal cells enabling further evaluation of bioavailability and nutrient uptake. One example is the human carcinoma cell line, Caco-2 cells, utilized to study the bioavailability of minerals [69]. Specifically, iron and zinc bioavailability from milk and soy based infant formulas has been studied using the Caco-2 cell lines [33]. Furthermore, this technique has also been used to study the intestinal assimilation of oxidized lipids in the presence of polyphenols [43]. Compared to human studies, this is a cost effective alternative, faster, and can be conducted in multiple samples as needed.

CHAPTER 2: RATIONALE AND HYPOTHESIS

The goal of this research was to determine whether the high fat induced calcium absorption observed *in vivo* can be partially explained by greater intestinal calcium bioaccessibility as measured by the TIM system. This information will shed light on the mechanism by which a high fat diet contributes to increased calcium bioaccessibility.

Data from our lab show a positive effect of chronic high fat diets on calcium absorption in a rodents [73] and in a cross sectional study in women [62] (Figure 2). However, in one study conducted in rodents calcium absorption was negatively affected with a high fat diet, but the model used in the study was a diet-induced obesity model fed a diet with a high fat content [75]. Different results in these studies could be attributed to the different age of the mice, or the extent of obesity possibly causing a leaky gut, influencing calcium absorption. In the study conducted in our lab, the mice were not obese and were fed a controlled intake to ensure that weight gain matched those mice consuming a normal fat diet [74]. Another study showed that the acute effects of fat on calcium absorption between three test meals consisting of low fat and regular ice cream (calcium fortified), and reduced fat milk did not differ [70]. However, the sample subjects used in the study was heterogeneous, given a fixed amount of food which could potentially have influenced the findings.

In addition, the influence of different fatty acids on membrane lipid composition and fluidity varies; this may be responsible for reducing the rate of calcium transport and absorption [27, 32]. In the present study, a low fat and two high fat formulas enriched in

MUFA and SFA and controlled for calcium were used to determine if different dietary fats in food is contributing to the up-regulation of intestinal calcium absorption in an *in vitro* intestinal model. We hypothesized that calcium bioaccessibility will be reduced with a more highly saturated diet because saturated FAs have a greater intestinal transit time, potentially giving rise to the formation of insoluble calcium soaps, hence reducing the calcium that is available for absorption.

Another goal of this project was to study the effect of pH on intestinal calcium bioaccessibility. A high pH occurs in patients with conditions of achlorhydria that is more prevalent with aging, diseases or with drugs used to treat gastritis or other GI disorders [40]. Therefore, to replicate conditions of achlorhydria in the TIM-1 system pH was increased in the stomach and intestinal segments and its effects on calcium bioaccessibility were examined. Previous studies posit that calcium absorption is compromised in patients with achlorhydria [58, 76]. When calcium was administered as calcium carbonate or citrate, calcium absorption from carbonate was lower in achlorhydric patients vs. normal and was similar between groups for calcium citrate. [58]. The study suggests that calcium carbonate is not the optimal dietary supplement in patients with achlorhydria. Although, different forms of calcium were not addressed in this study, the goal was to elucidate the mechanism contributing to the lower calcium absorption in this type of patients by examining the effect of pH and type of dietary fat on calcium bioaccessibility.

A secondary goal was to examine how much calcium is bioaccessible to the upper and lower portions of the intestine because there is evidence that calcium absorption is not restricted to the upper intestine, but also occurs in the lower portions [11, 22, 60]. It is also important to acknowledge that different fatty acids impact transit time through the intestine; for example, calcium can bind to SFA to form insoluble soaps thereby increasing fecal fat excretion. Hence, calcium bioaccessibility is expected to differ throughout the intestine. Also, whether pH and different FAs differentially affect site-specific calcium bioaccessibility was tested in this experiment.

The effect of chronic ad libitum fat intake on calcium absorption has been well studied. However, the effects of different types of fat, pH, and location in the GI tract on calcium bioaccessibility have never been addressed using a gastrointestinal model. This could play a pivotal role in identifying a potential mechanism for the up-regulation of calcium observed *in vivo.*

2.1 Specific Aims

- To determine if calcium bioaccessibility is influenced by the amount and type of fat using a standard Ca intake (500 mg/meal).
- To determine if calcium bioaccessibility is influenced by different levels of gastrointestinal pH (normal and high pH).
- To determine whether the amount and type of fat and pH affect calcium bioaccessibility at different locations (upper or lower portions) of the small intestine.

2.2 Hypothesis

It is hypothesized that dietary fat will increase calcium bioaccessibility. A high gastrointestinal pH to mimic achlorhydric conditions will decrease calcium bioaccessibility and location (jejunum and ileum) in the intestine will influence the results.

CHAPTER 3: MATERIALS AND METHODS

3.1 Gastrointestinal model of nutrient bioaccessibility

TIM-1 is an *in vitro* gastrointestinal model developed by the TNO Nutrition and Food Research Institute (Zeist, The Netherlands) (Figure 3). This multi-compartmentalized instrument consists of the stomach and three intestinal segments (duodenum, jejunum, and ileum) simulating the digestion process as it occurs in the small intestine in vivo systems [23]. This system ensures that *in vivo* factors such as meal size and duration, pH to sustain enzyme activity, peristaltic movements, nutrient and water absorption, gastric emptying, and intestinal transit times are emulated in this system [46, 47]. To mimic these digestive parameters, each compartment was filled with pre-specified amounts of pancreatic secretions, bile, and gastric secretions according to the fasted or fed states in the TIM-1 [65].

This research project was performed under the fed state and the following solutions were prepared: 7% pancreatin solution, small intestinal electrolyte solution, SIES (NaCl 5 g/L, KCl 0.6 g/L, $CaCl_2$ 0.3 g/L) and gastric electrolyte solution, GES (NaCl 6.2 g/L, KCl 2.2

g/L, $CaCl_2$ 0.3 g/L) [63]. Additionally, in preparation for feeding duodenal, jejunal, and ileal start residues were infused into each compartment before heating the machine to the appropriate physiological temperature of 37^{o} C [65].

3.2 Test meals

A low fat diet (LFD) and two high fat diets (HFD) enriched in monounsaturated (MUFA), and saturated fat (SFA) and controlled for calcium (500 mg) and other micronutrients (Research Diets, Inc. New Brunswick, NJ) were tested in a 5 hour experiment in triplicate for experiment 1 and in duplicate for experiment 2 The LFD has 75% of calories from carbohydrate, 15% of calories from protein, and 10% of calories from fat. The HFD was enriched with MUFA (39% carbohydrate, 15% protein, 46% fat) or SFA (37% carbohydrate, 19% protein, 44% fat) (Table 1).Ten grams of each test meal was packed into a tea bag to prevent excess digestive residue from clogging the machine and inserted into the gastric compartment. Prior to feeding, the gastric compartment was infused with: gastric start residue (5 g gastric enzyme solution and 11 mg amylase), 95 g gastric electrolyte solution, 150 g demi-water, and 50 g of water rinse. For the fed state protocol, the pH with a 40 minute half-life is programmed at 5.5 during feeding and decreases to 1.5 in the stomach with a 40 minute half-life and a gastric emptying rate with a 70 minute half-life [65]. During the course of digestion, gastric emptying, intestinal transit time, pH levels, and secretion fluid amounts are computer-controlled by the TIM-1 system [47]. The pH levels are maintained at 6.5, 6.8, 7.2 in the duodenal,

jejunal, and ileal compartments, respectively similar to the normal physiological conditions in a healthy individual [65].

3.3 Experiment 1 performed under normal gastrointestinal conditions

Experiment 1 was performed under normal physiological conditions after ingestion of the test diet (10 g) and in triplicates for the LFD, MUFA, and SFA meal matrices. Normal physiological conditions include the dynamics of gastric emptying, and intestinal transit times, the gastric and intestinal pH values, and the composition and activity of the secretion enzymes [47]. These experiments were carried out under the standard fed state program of the TIM-1 system.

3.4 Experiment 2 performed under high gastrointestinal pH conditions

The feeding conditions were similar to experiment 1, however, in experiment 2 the gastrointestinal pH was altered to mimic achlorhydric conditions (as seen in patients unable to produce HCl) or long term users of proton pump inhibitors (PPI) (Figure 4). In order to adjust and stabilize the pH at 7 throughout the gastrointestinal tract, a computer controlled program on the TIM-1 (FedLipidRUpH) was used to increase the pH.

3.5 Sampling collection

Dialysates from the jejunal and ileal compartments were collected (60, 120, 180, 240, 300 minutes) from the sampling bottles located at the bottom of the system. The total volumes for each compartment were measured and samples of 25 mL were taken and stored at -20^{o}C for analysis of calcium (bioaccessible components). The ileum effluent

(non-bioaccessible components) was also collected on ice (60, 120, 180, 240, 300 minutes).The volume of these samples were measured and 25 mL were collected and stored. The dialysates were analyzed for calcium bioaccessibility as described below.

3.6 Calcium analysis

A colorimetric method was used to measure calcium concentration in the TIM-1 dialysates from the three different intestinal regions (Pointe Scientific, Canton, MI, CV <4.6%). The colorimetric assay includes the color reagent (o-Cresolphthalein Complexone, CPC) and the buffer reagent (2-Amino-2-Methyl-1-Propanol). When calcium from the samples react with CPC, the color purple is produced, which is absorbed at a wavelength of 570 nm. This intensity of the color represents calcium concentration in samples. A microplate reader was utilized to read the results.

3.7 Statistical analysis

Statistical analysis was conducted using the SPSS package (IBM v23.0). Repeated measures ANOVA was performed to examine the interaction between diet and time on calcium bioaccessibility throughout the small intestine. When F ratio was significant, post-hoc analysis was conducted. Area under the curve (AUC) for each intestinal segment was calculated and one way ANOVA was used to compare between diets. Significance was considered at a p value < 0.05.

3.8 Summary of testing conditions

Three different meal matrices, varying in fat content and controlled for Ca (500 mg) and other micronutrients in 10 g of diet, were delivered into the TIM-1 instrument for a 5 hour digestion period. Testing conditions mimicked the fed state. TIM-1 parameters were set up at 37^{o}C and a normal physiological pH of 6.5, 6.8, and 7.2 for the duodenum, jejunum, and ileum, respectively for experiment 1(**normal gastrointestinal conditions**) and a steady pH of 7 throughout the GI tract for experiment 2 (**high gastrointestinal pH**). Jejunal, ileal, and ileal efflux dialysates were collected every hour (60, 120, 180, 240, 300 minutes) during the 5 hour digestion process. Experiment 1 was conducted in triplicate and experiment 2 in duplicate. Calcium that passes through the jejunal and ileal membranes is considered available for absorption, and therefore represents the amount of calcium that is bioaccessible. The calcium recovered from the ileal efflux (non-bioaccessible fraction) is considered the amount available to the large colon, and represents both the digested (unabsorbed) Ca and undigested Ca.

CHAPTER 4: RESULTS

4.1 CaB under normal gastrointestinal pH conditions

During normal gastrointestinal pH conditions, there was an increase in CaB by hour 1 for both diet groups (HFD and LFD); however, CaB was rose progressively for the HFD peaking at hour 3 while CaB remained stable for the LFD throughout the 5 hour digestion period (Figure 5). At normal gastrointestinal pH, CaB was significantly higher for the HFD (SFA+MUFA) compared to the LFD diet in the jejunum ($p = 0.001$).

Additionally, CaB changed significantly over time between diets in the jejunum ($p = 0.007$ for the interaction between time and diet) (Figure 5). Post hoc analysis indicated that CaB was greater for SFA compared to LFD and MUFA ($p \leq 0.01$) (Figure 6). In the ileum, there was an increase over time for CaB ($p \leq 0.01$); however, the difference between the diets did not reach statistical significance (Figure 6). Similarly, CaB during ileal efflux changed over time ($p = 0.001$), indicating that the majority of the non-bioaccessible fraction of calcium was available to the colon after 2 hours (Figure 7). The diets, however, did not significantly affect CaB in the ileal efflux (Figure 7). When calculating the percent of Ca intake utilized from the total calcium in the dialysates collected at every hour for the different meal matrices (LFD, MUFA, and SFA), CaB ranged from 8-12% in the jejunum and 4-9.5% in the ileum, under normal gastrointestinal conditions. Additionally, CaB ranged from 0-6.5% for the ileal efflux, the non-bioaccessible fraction of calcium for all diets. Calculation of AUC for calcium content showed that there was no significant difference for any diets in the jejunum, ileum, or ileal efflux.

4.2 CaB under high gastrointestinal pH

During high gastrointestinal pH conditions, CaB was not different when HFD (SFA+MUFA) was compared to LFD in the jejunum (Figure 8). Moreover, CaB did not change significantly over time or between diets in the intestinal segments (Figure 9). In the ileal efflux, there was a trend for non-bioaccessible calcium to increase over time ($p = 0.058$) (Figure 10), but similar to CaB in the jejunum and ileum, diets were not significantly different. In addition, CaB for the LFD, MUFA, and SFA diets ranged from

8.5-15% in the jejunum throughout the 5 hours of digestion while in the ileal intestinal segment, CaB varied from 3-9%. The non-bioaccessible fraction of calcium (ileal efflux) ranged from 0-3% for all the diets. Examining AUC indicated that CaB in the jejunum, ileum, and ileal efflux did not differ significantly for any of the diets.

In addition, when a comparison between CaB under normal gastrointestinal conditions vs increased gastrointestinal pH conditions was conducted, CaB in the jejunum was 90% lower under high gastrointestinal conditions ($p = 0.003$). Moreover, CaB in the ileum also differed between the two pH conditions and was 91% lower under high gastrointestinal pH conditions ($p = 0.036$). The ileal efflux was 94% lower under the high gastrointestinal conditions when compared to normal gastrointestinal conditions ($p = 0.001$) independently of the diets (Figure 11). When comparing the AUC between the high pH (7) and normal pH (6.5, 6.8, and 7.2) conditions, CaB was lower with a high gastrointestinal pH for all intestinal segments (jejunum, $p = 0.001$; ileum $p = 0.002$, and ileal efflux (0.002) (Figure 12).

CHAPTER 5: DISCUSSION

The TIM-1 is a computer-controlled, dynamic, multi-compartmental system that replicates the physiological activities occurring in the human stomach and small intestine [29, 48]. It has high predictive value because it mimics *in vivo* factors such as the secretion of digestive enzymes, adequate contents of cofactors and bile salts, buffer controlled pH levels, mixing by appropriate peristaltic movements, transit times, and the

ability to remove digestive products from each intestinal compartment [16]. The system is highly applicable in the field of nutrition and food science to study the absorption of vitamins and minerals [41, 72]. In this study, we examined the bioaccessibility of calcium in three different diets with varying fat contents at different sites of the intestine. In addition, we report differences in CaB in intestinal segments under two experimental conditions: normal gastrointestinal pH conditions (6.5, 6.8, and 7.2) and high gastrointestinal pH conditions (7) in the duodenum, jejunum, and ileum, respectively.

The findings in this study indicate that under normal gastrointestinal conditions, CaB was higher due to high fat intake (SFA and MUFA) or SFA alone in the jejunum compared to a diet that is lower in fat (LFD). High fat diets are associated with a greater production of insoluble Ca-fatty acid soaps. This relationship is evident in a large body of evidence [4, 15, 66] where greater calcium intake results in greater fecal fat excretion. In humans, the increase in fecal fat excretion is especially increased with SFA and this may be due to longer absorption time for SFAs in comparison to unsaturated FAs in the intestine [52]. In our study, the SFA diet were largely from coconut oil, that is mostly composed of lauric acid (C-12) [19] while the MUFA diet consisted largely of olive oil, which is high in oleic acid (C-18:1) (Table 1). Lauric acid in TAG occupies the sn-2 position [35] and remains in the monoacylglycerol form until absorption, decreasing its susceptibility to form soaps with calcium [48]. This may explain the unexpected higher CaB (possibly due to fewer Ca-fatty soaps) in the current study during the SFA diet. Also, the lipid source and type of SFA (beef tallow in the Denke study vs coconut oil in the current study) would also be expected to affect calcium bioaccessibility, absorption, and soap formation.

As FA chain length increases, absorption rates decrease. This occurs because long chain FAs have a melting point higher than body temperature and a higher tendency to form insoluble soaps with calcium [14, 35]. It is possible that these chemical properties of long chain FAs also contributed to the lower CaB for the MUFA than SFA diet after digestion in the TIM-1 instrument. Finally, because we only measured CaB and not absorption or fecal excretion, it is not possible to make conclusions about calcium beyond its bioaccessibility, and would need to be considered in another study.

Furthermore, a study conducted using the TIM system to examine the *in vitro* bioaccessibility of milk FAs and cis-9, trans-11 18:2 found that in addition to chain length, the degree of saturation also impacts fat digestion [26]. Gervais et al reported that as chain length increases from C-8 to C-18, there is a reduction in FA bioaccessibility; but only for SFAs [26]. Moreover, unsaturated FAs with one or more double bonds have greater bioaccessibility (and potentially absorbed more efficiently) than saturated FAs with the same number of carbons [26]. This study found that 14:1 and 16:1 showed greater bioaccessibility compared to their corresponding saturated FAs, 14:0 and 16.0 [26]. However, the same could not be reported for 18:0 and 18:1 because 18:0 showed the same or higher bioaccessibility compared to 18:1; therefore, more studies are warranted to explain this inconsistency.

Our findings are similar to *in vivo* studies conducted in mice showing increased fractional calcium absorption with the HFD enriched with saturated or monounsaturated fat [74]. This murine study was unique in that it examined the impact of different dietary fats on

calcium absorption in the absence of obesity. Also, the current study had similar macronutrient content as this mouse study [74] to determine calcium bioaccessibility. Hence, while we found that when both high fat diets increased CaB, similar to the absorption findings in the mice [74] we also found that CaB was highest with the SFA diet. Additionally, many clinical trials have also shown a positive effect of dietary fat on calcium absorption [57, 63, 75]. However, how the chemical properties of fat influence calcium absorption has not been addressed in human studies, and none have examined the interaction between fat type and calcium bioaccessibility or absorption.

In contrast to the jejunum, there was no interaction between diet type and CaB in the ileum. This is consistent with the evidence that FAs bind with calcium potentially making both less available for absorption in this segment. We also reported that CaB ranged from 8-12% in the jejunum and 4-9.5% in the ileum. These varied ranges in both intestinal segments may be indicative of the classic findings by Bronner et al who reported that in vivo the majority of calcium absorption occurs in the jejunum [9]. A previous study shows that approximately 10% of total calcium absorption takes place in the large intestine [9]. The ileal efflux in our study which represents the amount that is available for colon absorption was approximately 15% for the LFD and MUFA diets and 12% for the SFA diet and confirms the role of the large intestine in calcium absorption.

In addition to nutrient interactions influencing calcium absorption in humans, altered luminal factors such as intestinal pH can also influence absorption. Achlorhydric patients (absence of HCl secretion and high intestinal pH) have impaired calcium absorption [58,

75]. Also, persons taking proton pump inhibitors for gastritis, ulcers or other conditions would have similar high pH conditions in the gastrointestinal tract. When this condition was replicated in the TIM system, under high gastrointestinal pH conditions, CaB was significantly lower compared to normal conditions. Perhaps not surprisingly, there was no interaction between fat type and CaB in the jejunum, ileum, or ileal efflux under conditions of high pH which appears to mask any small dietary differences in CaB that occur under normal pH conditions. We did not address in this study, whether high gastrointestinal pH conditions affect fat bioaccessibility, but in humans fat absorption is impaired in achlorhydric patients [61]. Therefore, this would limit the ability to detect differences in CaB due to the type of fat.

Strengths and Limitations

TIM-1, an *in vitro* model of gastrointestinal bioaccessibility enables the evaluation of factors that can affect a nutrient prior to absorption. Nutrient bioaccessibility refers to the amount of compound that is ingested and potentially available for absorption [56]. This process relies on nutrient digestion and release from the meal matrix [18]. Such *in vitro* methods are valuable because they allow a step by step assessment of the events that lead to absorption, while *in vivo* studies provide the total amount of ingested compound that could be available for systemic circulation without consideration of chemical/physical factors that could potentiate a positive or negative effect on nutrient availability [69]. Because bioaccessibility is dependent on events preceding absorption in the intestinal lumen, the TIM-1 does not take into account biological parameters such as systemic metabolism [69]. Therefore, our findings may not be reflective of absorption as it occurs

in vivo, but allows us to determine whether the up-regulation of calcium absorption with a high fat diet as observed *in vivo* is due to greater calcium bioaccessibility from food. The absence of physiological and physiochemical events including the biological effects exerted by hormones and changes occurring in gut microflora and intestinal morphology as a result of calcium absorption cannot be assessed.

Calcium homeostasis *in vivo* is mediated by hormones including PTH and calcitonin. When calcium is low, PTH is responsible for calcium release from bone into extracellular fluid by inducing osteocytic bone resorption, thereby promoting bone remodeling [39]. In contrast, calcitonin has the opposite effects and inhibits the release of calcium and bone resorption when calcium is high [39]. In addition to hormones and transporters, calcium balance is significantly important in calcium regulation. The TIM-1 system is not a full representation of the intestinal environment as it lacks cell culture and hence, serves as a validation tool complementing in vivo studies [20]. One strength of our study was that experiment 1 was conducted in triplicates (compared to previous studies that used duplicate analysis) and this was done to establish the methods and confirm a low coefficient of variation (CV<8%). A limitation of the TIM-1 instrument is that it does not have the capacity to simulate the intricate physiological events that are occurring in the epithelial cells lining the intestinal tract [59]. In addition, the intestinal microflora of the upper GI tract is also absent in the TIM-1 model [59]. However, these were not goals in the current study design, but should be considered in future studies with various models, such as TIM-2. The TIM-2 instrument simulates the large intestine and would allow the study of the intestinal microflora and its interaction with calcium. This could have

implications for colonic absorption of calcium, as well as FAs and potentially other compounds.

A limitation of our study was that the protein content was slightly higher in the SFA diet compared to the MUFA and LFD diets. Studies have found that dietary protein intake plays a role in stimulating calcium absorption in the gut [25, 36]. Increased protein content in the test meals may have also contributed to the increased CaB found in the SFA diet in the jejunum. However, the difference in protein was very small (19 vs 15%) in the current study, relative to experiments indicating an effect of protein on Ca absorption. For example, in the Kerstetter study, the high protein group had nearly three times as much protein intake as the low intake group (135 vs 45 g/d) [36]. The protein content for the diets used in our study was within normal range [74] whereas, the Kerstetter study had a high protein content which may have caused the increase in calcium absorption [36]. Another limitation was the carbohydrate contents of the diets: corn starch, maltodextrin, and sucrose levels were varied. The LFD and MUFA contained significant amounts of both corn starch and maltodextrin while the SFA contained none. Sucrose, on the other hand, was higher in the SFA, 396 g/kg diet, compared to 98 and 34 g/kg diet in the LFD and MUFA, respectively. Recent studies suggest that carbohydrates such as lactose may play a role in enhancing intestinal calcium absorption [3, 28]. Although current literature suggests a positive effect of lactose on calcium absorption, research on the effects of corn starch, maltodextrin, and sucrose are lacking. Therefore, varied amounts of these carbohydrates present in the test diets would not contribute to differences in our findings.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The future directions for this study might include the use of Caco 2 cells with similar physiological functions of intestinal enterocytes to extend our findings on the bioavailability of calcium. It is possible to predict calcium absorption, as occurs in vivo, by inoculating Caco-2 cells with TIM-1 jejunal, ileal, and ileal efflux dialysates because Caco 2 cells can reproduce the physical, chemical, and biological mechanisms and events taking place in the small intestine during absorption [20]. This could be assessed under both normal conditions and under abnormal conditionals that represent the pathophysiology associated with GI disorders or use of medications, such as PPIs. Moreover, our experiments were performed under the fed state and it would be interesting to see how CaB would be affected under fasted conditions.

To conclude, the HFD showed higher CaB compared to the LFD in the jejunum and CaB further differed due to the type of FA. There was higher CaB with both high fat diets which is consistent with studies of absorption in mice and humans; however CaB was highest with the SFA diet in the jejunum. While only the SFA diet showed higher CaB and this might be consistent with findings in humans showing an association between high fat intake and Ca absorption [62, 74], since SFA intake is generally high in the Western diet [13]. However, when we examined whether the type of fatty acid affects Ca absorption in mice [72], there was no difference in Ca absorption between SFA and MUFA diets. Overall, because we found higher CaB with the HFD, this indicates that CaB can partially explain the higher Ca absorption associated with a high fat diet observed in vivo and we

speculate that there are unique interactions between the SFA meal matrix and CaB in the jejunum.

Finally, CaB was significantly reduced at high gastrointestinal pH compared to normal gastrointestinal conditions and would have implications in a large population of patients with gastrointestinal disorders. This might contribute to the low bone mass and higher risk of osteoporosis in this patient population and our findings that CaB is dramatically reduced under higher pH conditions indicate the urgent need for future studies.

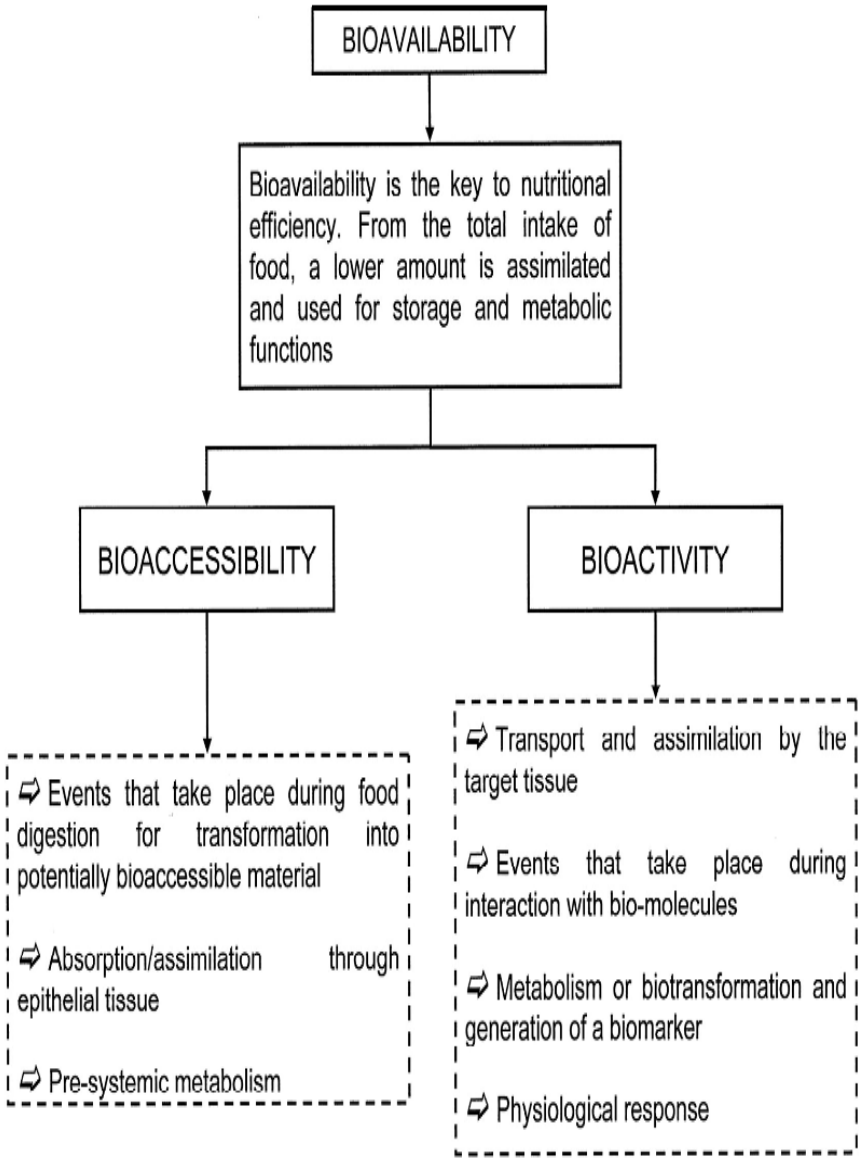


Figure 1: Flow diagram explaining the relationship between bioavailability, bioaccessibility, and bioactivity.

Adapted from Nutrition Research, Fernandez-Garcia et al. In vitro bioaccessibility assessment as a prediction tool of nutritional efficiency, Nutrition Research. 2009;11:751-760.

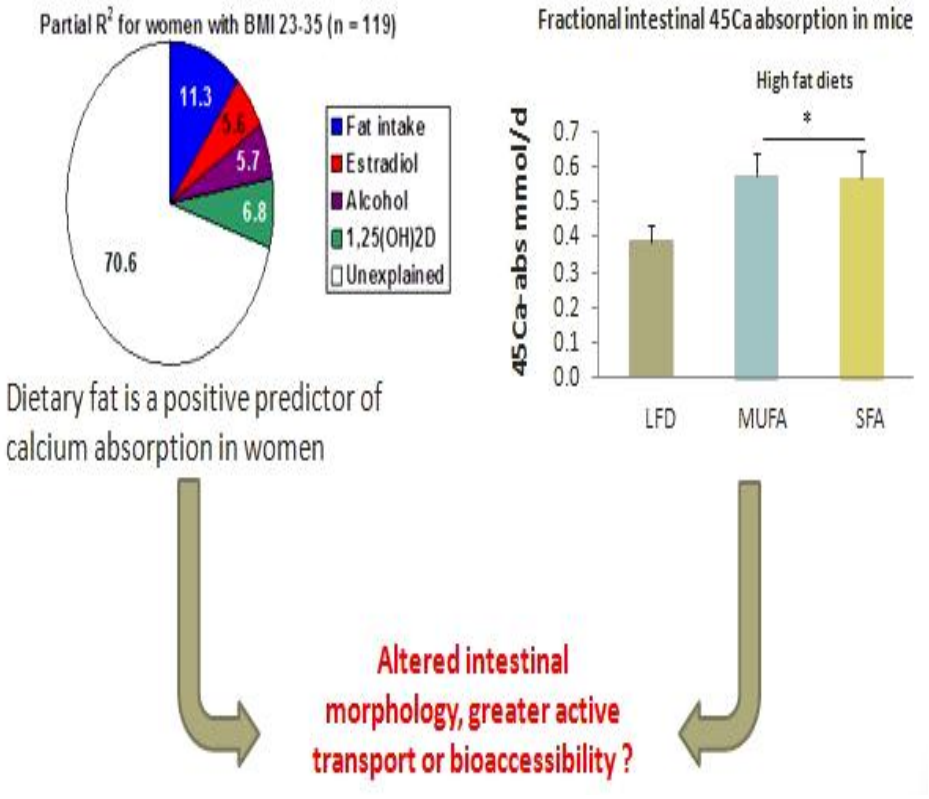


Figure 2: Data from our lab showing greater calcium absorption with increased dietary fat intake in women[65] and mice[76]

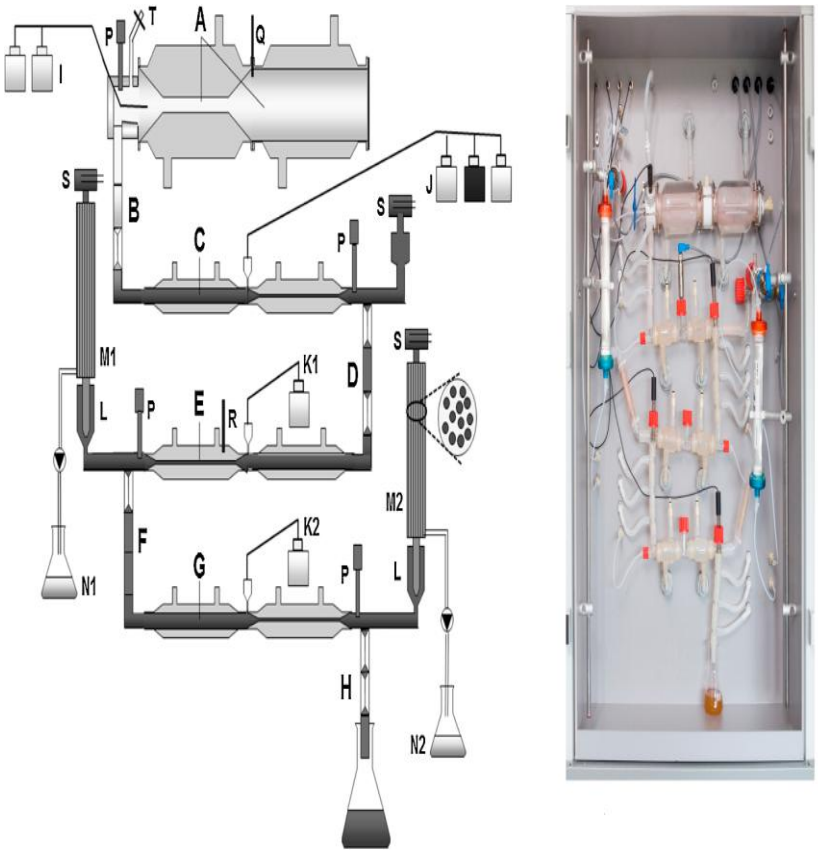


Figure 3: Schematic of the TNO gastrointestinal model (TIM-1)[73]
TIM-1 replicates the conditions of the small intestine: (A) gastric compartment and three intestinal segments (C) duodenum, (E) jejunum, (G) ileum. These segments are interconnected by peristaltic valves which control (B) gastric emptying, (D and F) intestinal transit, (H) ileal emptying.

Adapted from International Journal of Pharmaceutics, Verwei et al., Evaluation of two dynamic *in vitro* models simulating fasted and fed state conditions in the upper gastrointestinal tract (TIM-1 and tiny-TIM) for investigating the bioaccessibility of pharmaceutical

Table 1- Dietary composition and energy content of test meals

Ingredient	LFD	MUFA	SFA
		g/kg diet	
Casein	146	183	200
L-cysteine	3	4	0
DL-methionine			3
Corn starch	540	249	0
Maltodextrin	98	189	0
Sucrose	98	34	396
Cellulose	49	61	50
Soybean oil	6	36	45
Coconut oil	0	0	135
Lard	11	64	0
Olive oil	26	153	0
Mineral mix	10	12	35
Vitamin mix V10001	10	12	10
Choline bitartrate	2	2	2
Energy, kcal/g diet	3.9	4.8	4.6
% Energy			
Carbohydrate	75	39	37
Fat	10	46	44
Saturated (%kcal of fat	20	20	41
Monounsaturated (%kcal of fat)	60	60	41
Polyunsaturated (%kcal of fat)	20	20	18

LFD, low fat diet; MFD, monounsaturated fat diet; SFA, saturated fat diet
Prepared by Research Diets Inc, New Brunswick
Protein intake ranged from 15-19% in all diets
Calcium 50mg/g diet for all test meals

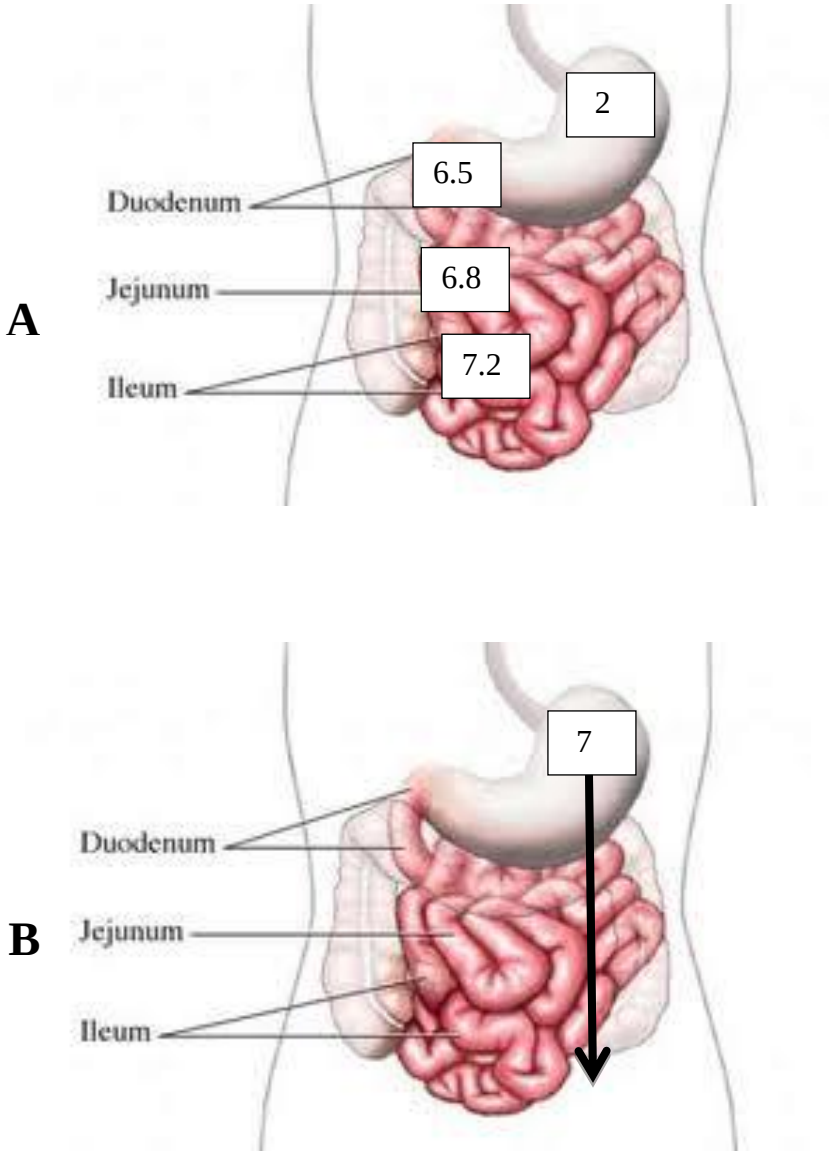


Figure 4: Gastrointestinal pH conditions for experiments 1 and 2

A. Gastric, duodenal, jejunal, and ileal pH are 2, 6.5, 6.8, and 7.2, respectively at normal gastrointestinal conditions **(Exp. 1).**
B. Gastric, duodenal, jejunal, and ileal pH were manipulated to be 7 throughout the intestinal tract to mimic achlorhydric conditions

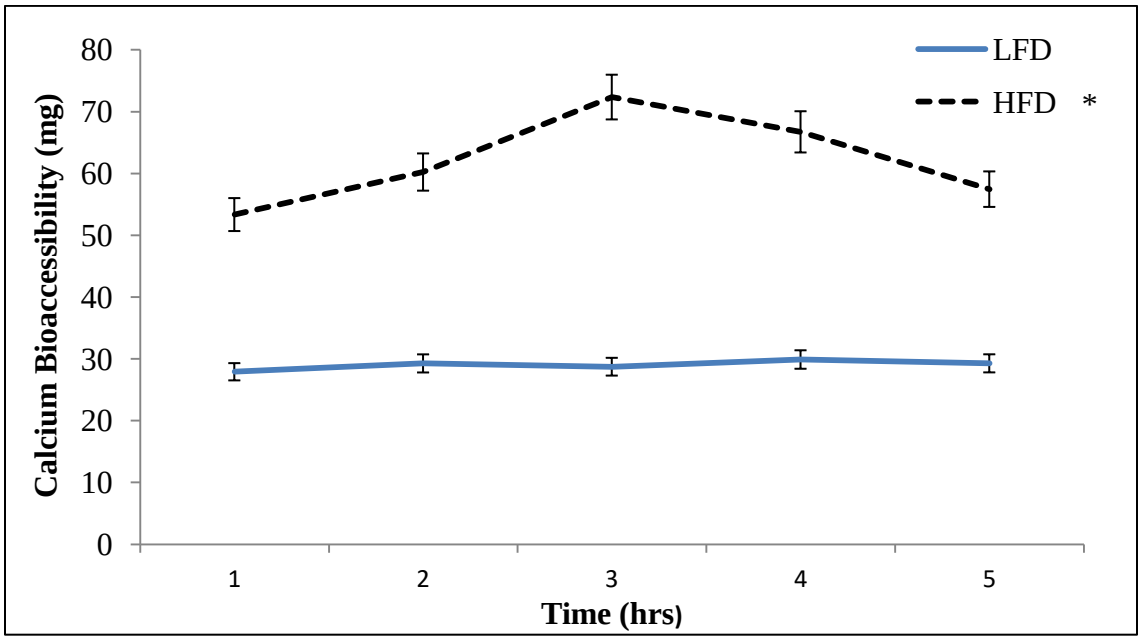


Figure 5: Hourly calcium bioaccessibility in the small intestine over a 5 hour digestion period during normal gastrointestinal conditions.
***** CaB differs for the HFD (MUFA and SFA combined) as compared to the LFD diet ($p = 0.001$)

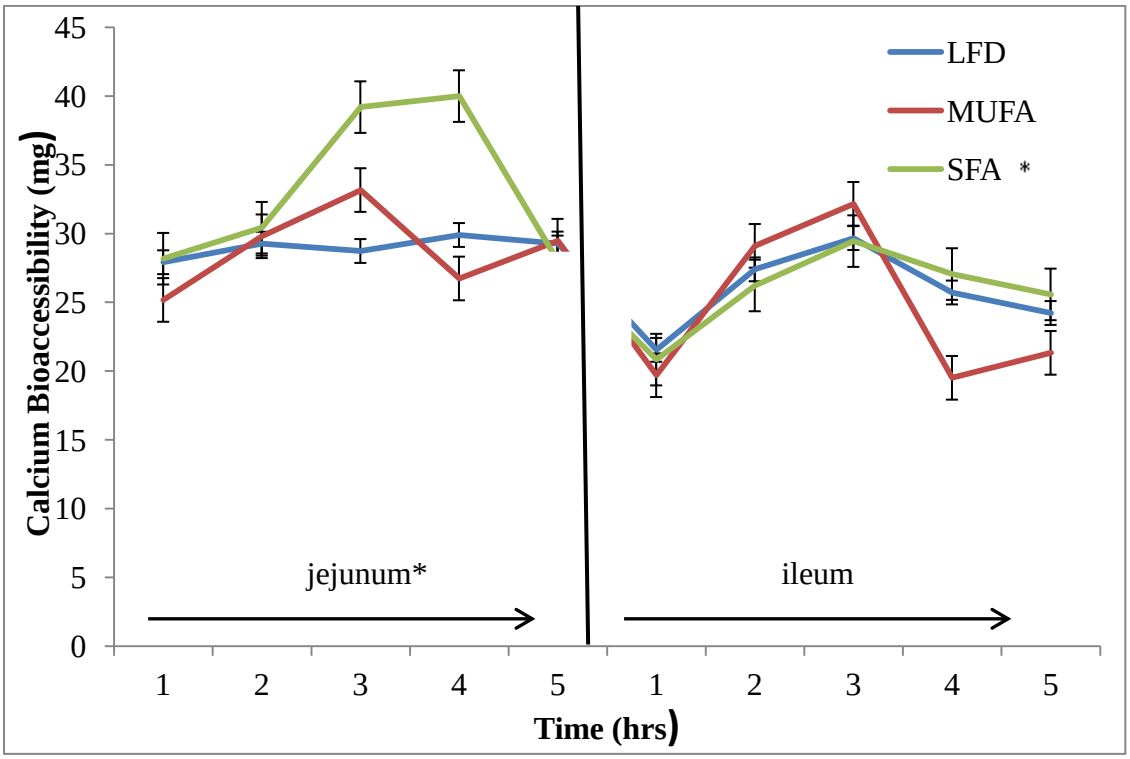


Figure 6: CaB in the jejunum and ileum for LFD, MUFA, and SFA during normal gastrointestinal pH conditions

* $p = 0.001$ for the interaction between time and diet. Tukey's post hoc analysis shows that CaB for the SFA diet was different compared to LFD and MUFA ($p \leq 0.001$)

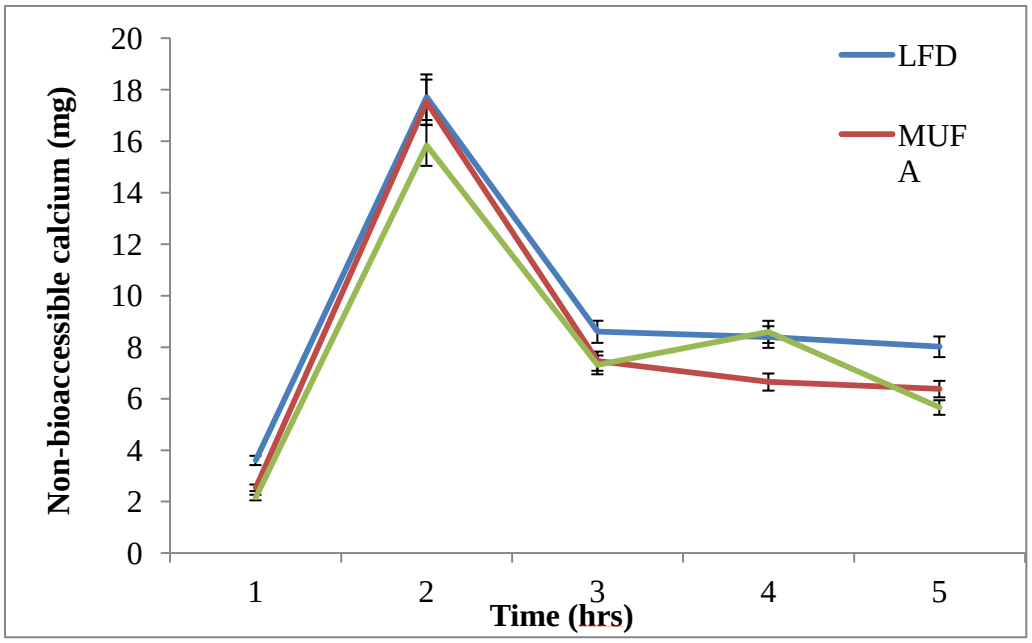


Figure 7: Non-bioaccessible calcium (ileal efflux) during normal gastrointestinal pH conditions

No significant interaction observed between diet type and mean hourly non-bioaccessible calcium

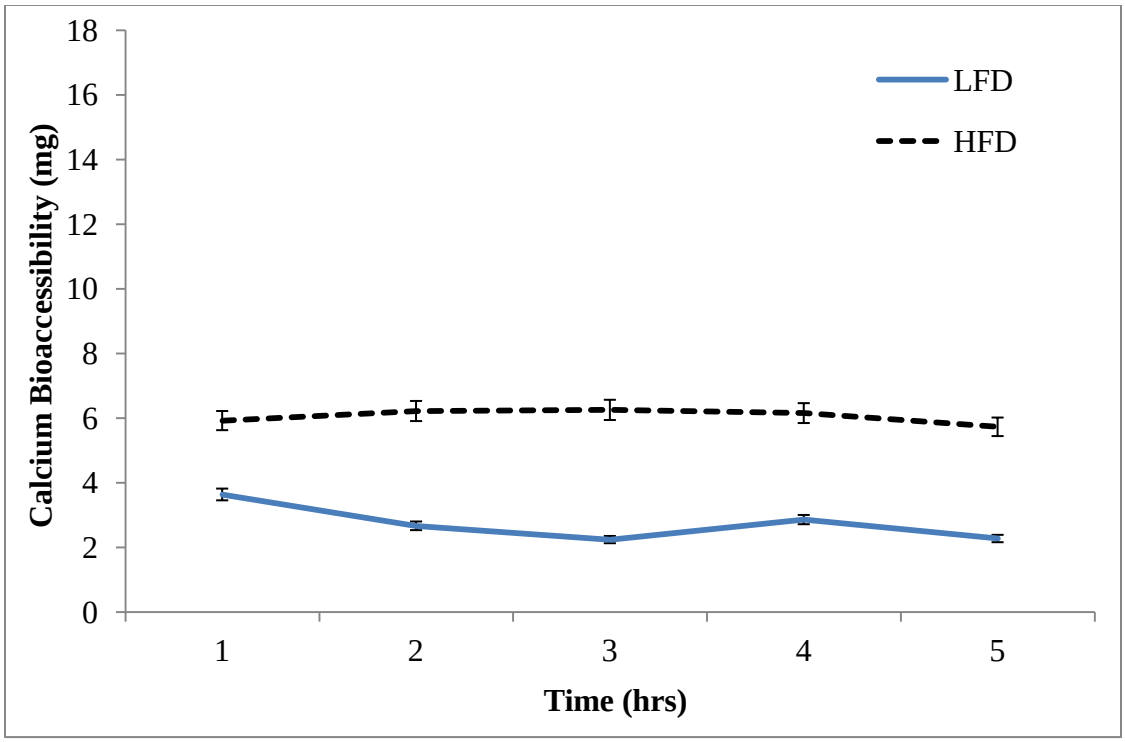


Figure 8: Hourly calcium bioaccessibility in the jejunum during high pH gastrointestinal conditions.
CaB did not differ for the HFD (SFA and MUFA diets combined) and LFD diets.

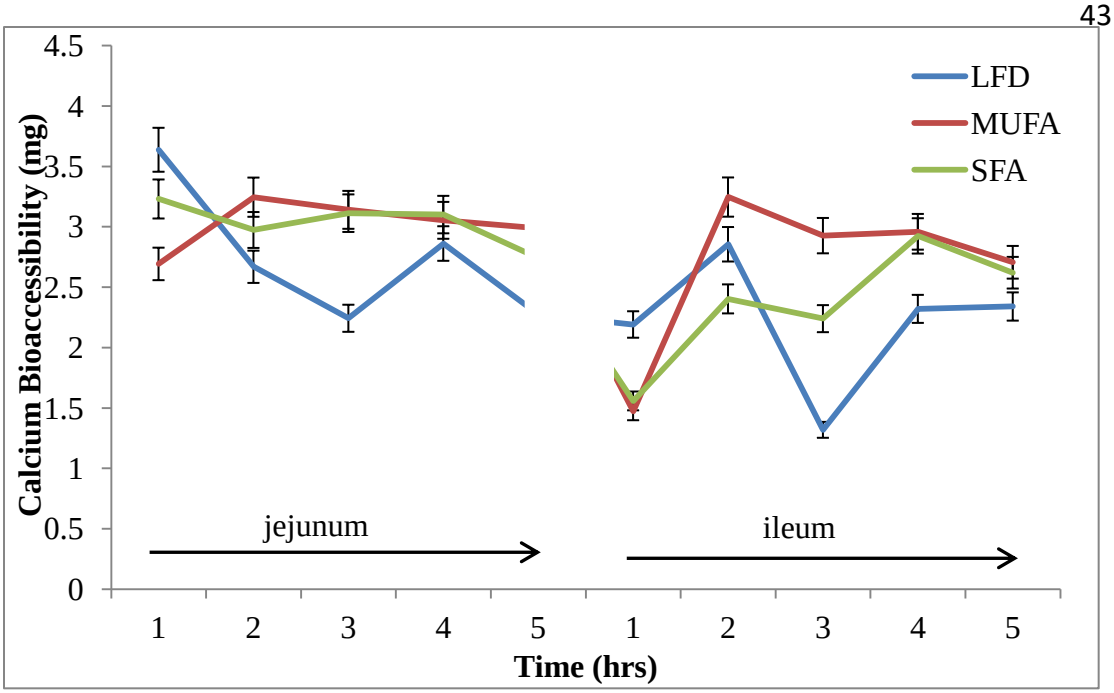


Figure 9: CaB in the jejunum and ileum for LFD, MUFA, and SFA during high gastrointestinal pH conditions
CaB did not differ for any of diets in the intestinal segments

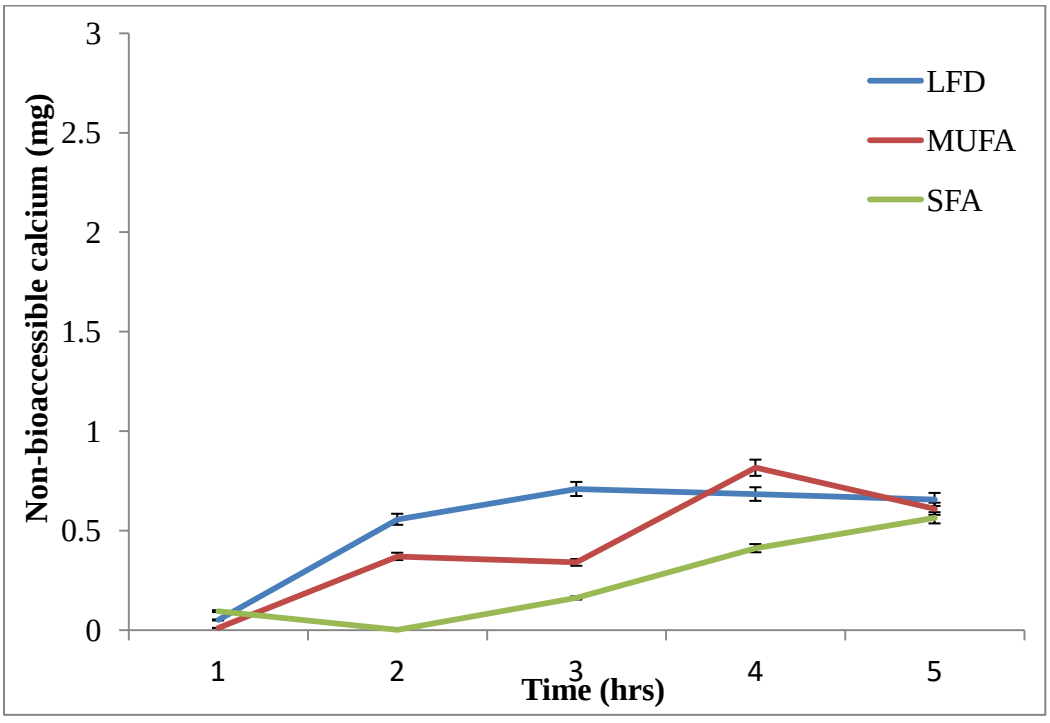


Figure 10: CaB in the jejunum and ileum for LFD, MUFA, and SFA during high gastrointestinal pH conditions
CaB did not differ for any of diets in the intestinal segments

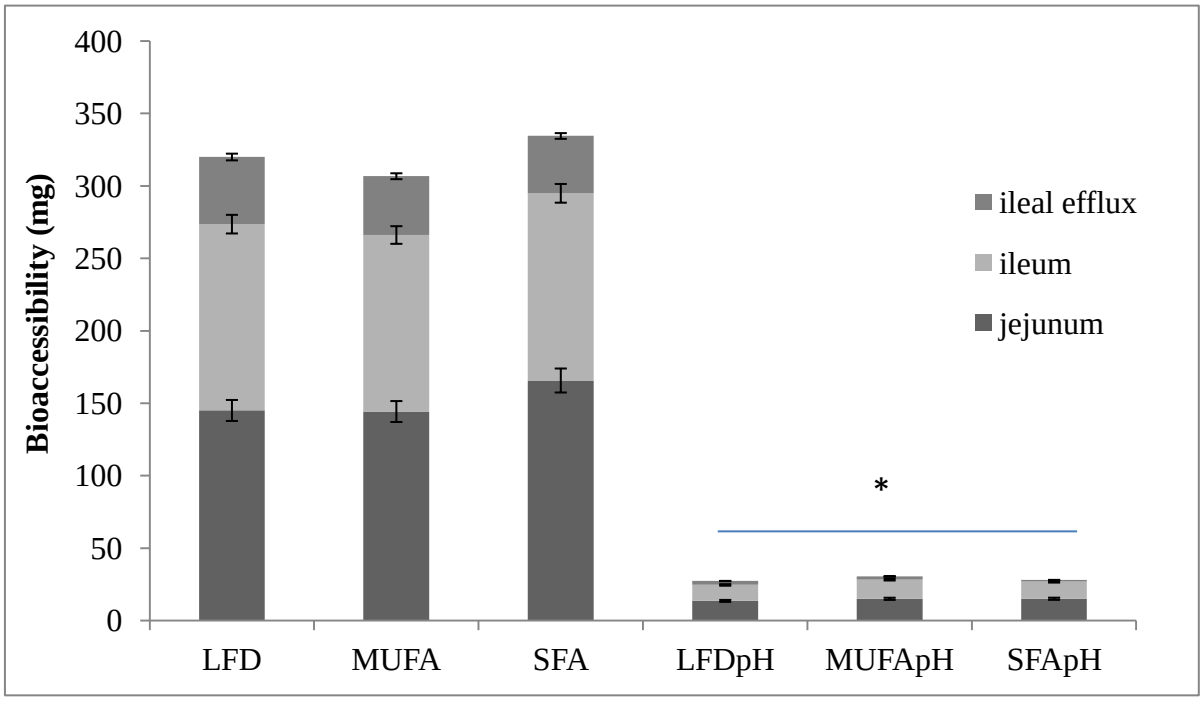


Figure 11. Total bioaccessible and non-bioaccessible calcium in different intestinal segments under normal and high pH gastrointestinal conditions.
* CaB differs between high pH and normal gastrointestinal conditions independent of diets in the; jejunum (p = 0.003), ileum (p = 0.036), and ileal efflux (p =0.001).

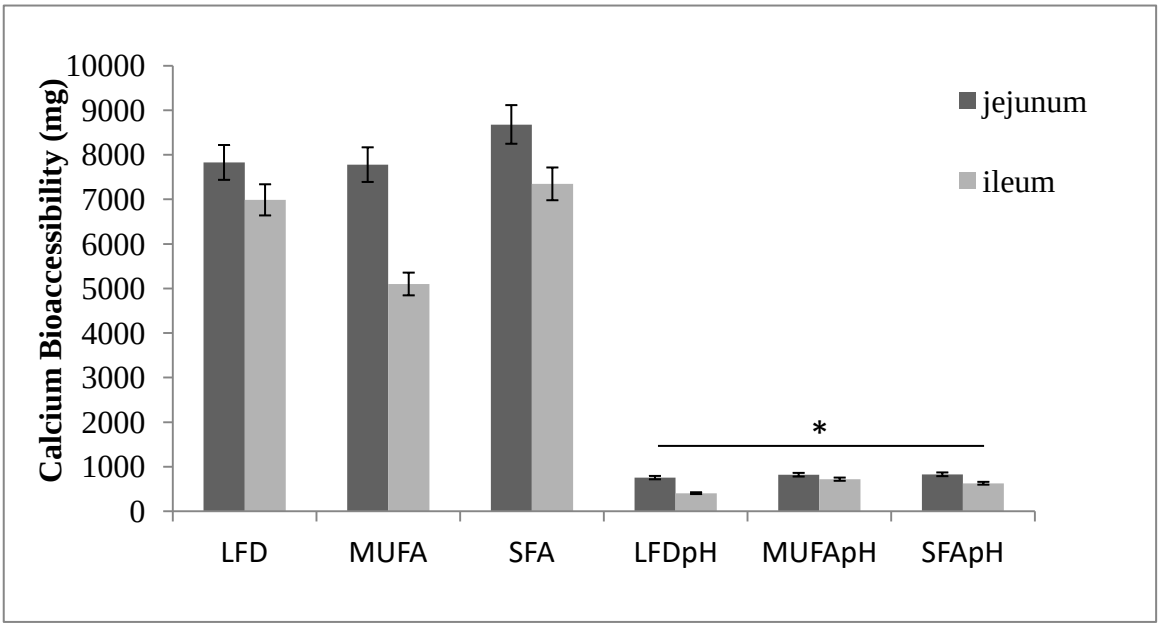


Figure 12. AUC for bioaccessible and non-bioaccessible calcium in different intestinal segments under normal and high pH gastrointestinal conditions.
* AUC differs in the high pH compared to normal gastrointestinal conditions independent of diets; jejunum (p = 0.001), ileum (p = 0.002)

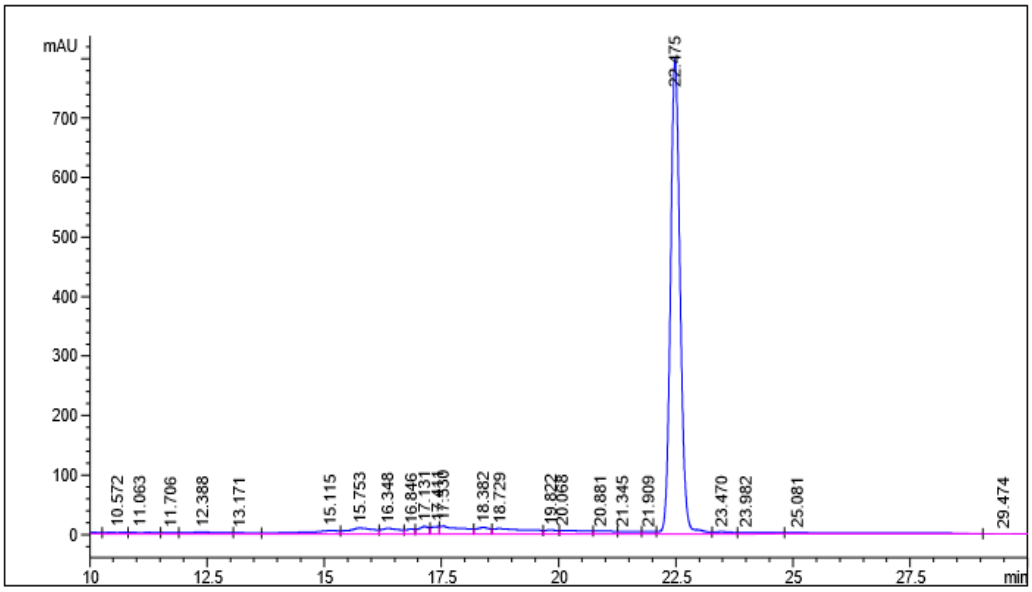
APPENDIX I: Vitamin D analysis of TIM-1 jejunal, ileal, and ileal efflux dialysates

Objective: To determine the concentration of vitamin D_3 in the jejunal, ileal, and ileal efflux dialysates from the TIM-1 instrument with high compared to low fat diet.

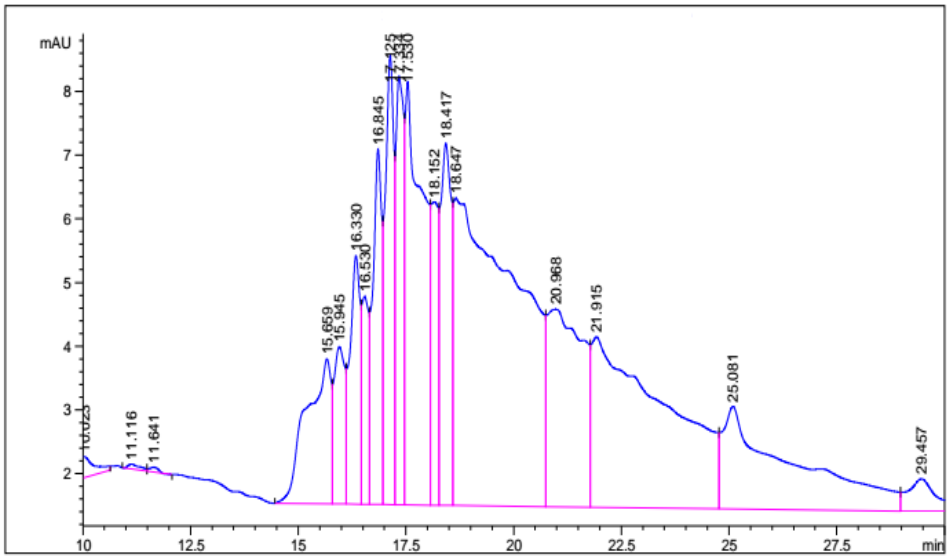
Hypothesis: Vitamin D3 would show higher bioaccessibility with a high fat diet and explain the greater rise in serum 25-hydroxyvitamin D levels with high fat intake [48].

Method: The quantification of vitamin D_3 was conducted using a reversed phase-high performance liquid chromatography (HPLC) and UV detector (Agilent technology) procedure that was developed for vitamin D_3 and vitamin D_2 analysis [49] and is considered simple and reliable as it does not involve pre-treatment, extraction and sample purification steps. A stock solution mixture of vitamin D_3 standard and an internal standard (I.S) vitamin D_2 (1 mg/ml) in methanol was prepared and stored at -20 ^{o}C. In the original protocol published [49], the samples were solid and were prepared in the mobile phase; for our study the samples were in the liquid form and were prepared using methanol and hexane or heptane was used to extract the vitamin D_3. These samples with the added methanol and hexane or heptane were shaken twice and centrifuged for 10 minutes. Afterwards, the supernatant was filtered and N_2 was used to evaporate the samples until dry.

Results: The vitamin D concentration in the jejunal, ileal, and ileal efflux samples throughout the 5 hour digestion period was undetectable with this protocol for both the high fat diet and low fat diets. We did observe peaks for the LFD diet in the ileum at hour 1 (Figure A), but consequent trials did not show the same results. It is concluded that an experimental protocol needs to be further developed to measure the vitamin D_3 concentrations in TIM-1 dialysates and should be addressed in future studies.



(a)



(b)

Figure A: Vitamin D_3 peak in ileum during (a) successful (b) and unsuccessful HPLC run.

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