JÚNIA MARIA GERALDO GOMES

INCREASED FAT-FREE MILK CONSUMPTION IMPROVES WEIGHT LOSS, BODY COMPOSITION, METABOLIC SYNDROME, AND CARDIOMETABOLIC OUTCOMES IN ADULTS WITH TYPE 2 DIABETES MELLITUS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS-BRASIL
2016

Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV

T

G633i
2016

Gomes, Júnia Maria Geraldo, 1983-

Increased fat-free milk consumption improves weight loss, body composition, metabolic syndrome, and cardiometabolic outcomes in adults with type 2 diabetes mellitus / Júnia Maria Geraldo Gomes. - Viçosa, MG, 2016.

xiv, 140f. : il. ; 29 cm.

Inclui apêndice.

Orientador: Rita de Cássia Gonçalves Alfenas.

Tese (doutorado) - Universidade Federal de Viçosa.

Inclui bibliografia.

1. Diabéticos. 2. Diabetes. 3. Síndrome metabólica. 4. Dieta - Leite. 5. Pesos e medidas. I. Universidade Federal de Viçosa. Departamento de Nutrição e Saúde. Programa de Pós-graduação em Ciência da Nutrição. II. Título.

CDD 22 ed. 616.462

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APROVADA: 22 de dezembro de 2016.

_____	_____
Maria Sonia Lopes Duarte	Fernanda de Carvalho Vidigal
_____	_____
Raquel Duarte Moreira Alves	Hércia Stampini Duarte Martino (Coorientadora)

_____

Rita de Cássia Gonçalves Alfenas
(Orientadora)

"Vinde a mim, todos os que estais cansados e sobrecarregados, e eu vos aliviarei. Tomai sobre vós o meu jugo e aprendei de mim, porque sou manso e humilde de coração; e achareis descanso para a vossa alma. Porque o meu jugo é suave, e meu fardo é leve."

(Mateus, 11:28-30)

Esta tese é dedicada a Deus, aos meus pais José Geraldo e Marlene, ao meu marido Arlindo, às minhas irmãs Filomena e Juliana, às minhas sobrinhas Mariana e Bianca, ao meu amigo Jorge e à professora Rita.

AGRADECIMENTOS

A Deus, por me iluminar e colocar em meu caminho tudo o que preciso para cada momento.

Ao meu marido e companheiro, que sempre me apoiou nas minhas decisões e que fez inúmeros sacrifícios para que eu chegasse até aqui. Agradeço a dádiva de compartilhar a vida com você, por toda sua dedicação e por preencher meus dias com carinho e alegria. Obrigada, Arlindo, por seu amor!

À minha querida orientadora, Profª. Rita Alfenas, pela confiança que depositou em mim, pela amizade, carinho e paciência. Aprendi muito com sua ética, profissionalismo e, sobretudo, respeito às pessoas. Você é o exemplo perfeito do que a palavra “Mestre” representa.

À minha família, sem a qual eu nada seria: seu amor incondicional me ensinou o sentido da vida! Meus amados pais, obrigada por acreditarem em mim e por toda dedicação. Às minhas irmãs, amigas e conselheiras leais. Minhas lindas sobrinhas, que tornam minha vida mais leve. Minha sogra e minha cunhada, minha segunda família que aprendi a amar.

Ao meu irmão de coração, Jorge de Assis Costa, agradeço o privilégio de ser sua amiga, a cumplicidade e todo apoio durante toda a realização deste trabalho.

Às minhas coorientadoras, Profas. Ana Paula Boroni Moreira e Hércia Stampini Duarte Martino, pelo apoio e ajuda oportuna.

À profª. Dionéia Evangelista César (UFJF), pelo aprendizado e por me acolher de forma tão gentil e receptiva.

Às Profas. Eliana Gomes, Fernanda Vidigal, Raquel Moreira, Maria Sônia Duarte e Júlia Carraro, por prontamente aceitarem compor a banca e pelas contribuições acerca desta tese.

Aos estagiários, às bolsistas e à técnica de enfermagem Jéssica Louzada,

que auxiliaram na concretização desta pesquisa com disponibilidade, carinho e dedicação.

Aos voluntários, agradeço por compartilhar conosco suas vivências, pela disposição e compromisso.

À Universidade Federal de Viçosa (UFV), Departamento de Nutrição e Saúde (UFV) e ao Instituto Federal do Sudeste de Minas Gerais Câmpus Barbacena, que proporcionaram meu aprendizado.

Aos professores e técnicos administrativos do Departamento de Nutrição e Saúde (UFV), que sempre foram muito solícitos.

Aos colegas do Instituto Federal Câmpus Barbacena, em especial aos professores do curso de Nutrição, ao diretor-geral José Alexandrino Filho e à Profª. Renata de Souza Ferreira, pela compreensão e amizade.

Às equipes do Laboratório de Estudos em Ingestão Alimentar – LEIA (DNS/UFV), Laboratório de Metabolismo e Composição Corporal - LAMECC (DNS/UFV), Laboratório de Análises Clínicas - LAC (DNS/UFV), Laboratório de Ecologia e Biologia Molecular de Microrganismos – LEBIOMM (UFJF), Microbiologia de Anaeróbios (DMB/UFV) e Laboratório de Analises Clínicas da Divisão de Saúde (UFV), pelos ensinamentos e pelo convívio agradável.

Aos colegas da Pós-Graduação em Ciência da Nutrição (UFV), com os quais compartilhei os prazeres e dificuldades desta jornada.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pelo apoio financeiro concedido.

A todos que contribuíram para a realização deste trabalho, meu MUITO OBRIGADA!

BIOGRAFIA

Júnia Maria Geraldo Gomes nasceu em 17 de julho de 1983 no município de Ouro Preto (MG).

Em maio de 2002, iniciou o curso de graduação em Nutrição na Universidade Federal de Viçosa (MG), concluindo-o em outubro de 2006. Neste mesmo mês, ingressou no mestrado do Curso de Pós-Graduação *Stricto Sensu* em Ciência da Nutrição da Universidade Federal de Viçosa (MG), obtendo o título de mestre em outubro de 2008, com a dissertação intitulada “Impacto do índice glicêmico e da qualidade da dieta nos marcadores inflamatórios associados ao diabetes *mellitus* tipo 2”.

Durante 2008 e 2009, foi professora substituta na Universidade Federal de Ouro Preto (MG), onde coordenou o estágio em Nutrição Social e lecionou as disciplinas Patologia da Nutrição, Estágio Supervisionado em Nutrição Clínica, Nutrição da Criança e do Adolescente, Estágio Supervisionado em Nutrição Social e Introdução ao curso de Nutrição.

Desde fevereiro de 2010, atua como docente efetiva no Instituto Federal do Sudeste de Minas Gerais (IF Sudeste MG). Foi coordenadora do curso técnico em Nutrição e Dietética (2010-2012) e do curso Superior de Nutrição (2012-2013), orientadora de projetos de extensão e de iniciação científica, e lecionou as disciplinas Nutrição e Saúde, Nutrição Básica, Nutrição do Idoso, Planejamento de Refeições, Patologia da Nutrição e Dietoterapia I, Tecnologia de Alimentos, Estágio Supervisionado em Nutrição Clínica, Avaliação nutricional, Introdução à nutrição, Ética profissional, Nutrição Materno-Infantil e Metodologia Científica.

Em maio de 2013, ingressou no doutorado do Curso de Pós-Graduação *Stricto Sensu* em Ciência da Nutrição da Universidade Federal de Viçosa (MG) e pleiteou o título de doutora em dezembro de 2016.

LISTA DE ABREVIATURAS

ADA	*American Diabetes Association*
ApoA-I	*Apoprotein A-I*
BA	*Bile acid*
BCAA	*Branched chain amino acid*
BMC	*Bone mineral content*
BMI	*Body Mass Index*
BP	*Blood pressure*
BPI	*Bactericidal/ permeability-increasing protein*
Ca	*Calcium*
CaP	*Calcium phosphate*
CD	*Low-Ca control diet*
CD14	*Cluster of differentiation 14*
CI	*Confidence interval*
Cr-EDTA	*EDTA chromium*
CRM	*Cardiometabolic risk factors*
CRP	*C-reactive protein*
CV	*Coefficient of variation*
DBP	*Diastolic blood pressure*
DRIs	*Dietary Reference Intakes*
EER	*Estimated Energy Requirement*
FAS	*Fatty acid synthase*
FFM	*Free-fat mass*
FM	*Fat mass*
FW	*Faecal water*
GIP	*Glucose-dependent insulinotropic polypeptide*
GLP	*Glucagon-like peptide*
Hb1Ac	Glycated hemoglobin
HDL-c	*High-density lipoprotein cholesterol*
HipC	*Hip circumference*
HOMA	*Homeostasis Model Assessment*
HOMA2-B%	*HOMA2 - beta-cell function*
HOMA2-S%	*HOMA - Insulin sensitivity*
HOMA-IR	*Homeostasis Model Assessment of Insulin Resistance*
IGT	*Impaired glucose tolerance*
II	*Intestinal integrity*

IPAQ	*International Physical Activity Questionnaire*
IR	*Insulin resistance*
LAL	*Limulus amebocyte assay*
LAP	L*ipid accumulation product*
LBP	*Lipopolysaccharide-binding protein*
LDL-c	*Low-density lipoprotein cholesterol*
LPS	*Lipopolysaccharide*
MD	*Fat-free milk diet*
MetS	*Metabolic Syndrome*
Mg	*Magnesium*
MUFA	*Monounsaturated fatty acid*
NEI	*Nutrient and energy intake*
NEFA	*Non-esterified free fatty acid*
OTU	*Operational taxonomic unit*
P	*Phosphorus*
PAL	*Physical activity level*
PTH	*Parathyroid hormone*
PUFA	*Polyunsaturated fatty acid*
QFFQ	*Quantittative food frequency questionary*
RCT	*Randomized clinical trial*
rEI	*Reported energy intake*
RSG	*Rosiglitazone*
SBP	*Systolic blood pressure*
SCFAs	*Short chain fatty acids*
SFA	*Saturated fatty acid*
T2DM	*Type 2 diabetes mellitus*
TEE	*Total energy expenditure*
TG	*Triglycerides*
TLR	Toll-like receptor
UCP-2	*Uncoupling protein 2*
WC	*Waist circumference*
WHR	*Waist-hip ratio*
Zn	*Zinc*

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ABSTRACT

GOMES, Júnia Maria Geraldo, D.Sc., Universidade Federal de Viçosa, December, 2016. I**ncreased fat-free milk consumption improves weight loss, body composition, metabolic syndrome, and cardiometabolic outcomes in adults with type 2 diabetes mellitus.** Adviser: Rita de Cássia Gonçalves Alfenas. Co-advisers: Ana Paula Boroni Moreira and Hércia Stampini Duarte Martino.

INTRODUCTION: It has been suggested that the consumption of fat-free dairy and of dietary calcium (Ca) seem to act on glycemic and body weight control, favoring type 2 diabetes mellitus (T2DM) prevention and treatment. However, to our knowledge, the effects of increased fat-free milk consumption on metabolic control of individuals with T2DM have not been assessed in any other study. **OBJECTIVE:** To evaluate the effects of fat-free milk consumption on body weight, body composition, metabolic syndrome (MetS) components, and cardiometabolic outcomes in aldults with T2DM. **METHODS:** Two randomized crossover clinical trials (N = 14) (Articles 1 and 2), and one randomized parallel design clinical trial (N = 36) (Article 3) were performed. Subjects with T2DM and low habitual Ca consumption (< 600 mg/d) participated of two 12-week experimental phases: high-Ca fat-free milk (MD) or low-Ca control diet (CD). In the crossover studies (Articles 1 and 2) there was an 8-week washout between experimental phases. Subjects daily consumed a breakfast shake containing 700 mg of Ca from fat-free milk (MD) (~3 servings of fat-free milk) or 6.4 mg of Ca (CD). Energy restricted diets (-500 kcal/d) containing 800 mg of Ca/d were prescribed. **RESULTS: Article 1: Fat-free milk enhances weight loss, improves body composition, and promotes glycemic control in adults with type 2 diabetes *mellitus* – a randomized clinical trial -** Dietary records data indicated the consumption of 1,200 mg of Ca/d during MD and of 525 mg of Ca/d during CD. There was a greater reduction in body weight, body fat mass, waist circumference (WC) and waist-hip ratio after MD. Serum 25-hydoxyvitamin D and HOMA2-B% increased and serum uric acid, parathormone, and glycated hemoglobin concentrations reduced after MD. The consumption of approximately 3 servings of fat-free milk (700 mg of additional Ca) enhanced weight loss, improved body composition, and promoted glycemia control in individuals with T2DM and low habitual Ca consumption. **Article 2: Increased fat-free milk consumption, metabolic syndrome, and**

cardiometabolic outcomes in adults with type 2 diabetes *mellitus.* There was a greater reduction in WC, systolic (SBP) and diastolic blood pressure (DBP), and lipid accumulation product (LAP) index after MD compared with CD. Only MD phase increased HDL (high-density lipoprotein cholesterol) / LDL (low-density lipoprotein cholesterol) ratio and decreased total cholesterol, LDL-c, SBP and DBP, and LAP index. We conclude that the consumption of approximately 3 servings of fat-free milk (700 mg of additional Ca) decreased some MetS components and cardiometabolic measures adults with T2DM. **Article 3: Dietary calcium from dairy, body composition and glycemic control in patients with type 2 diabetes: a parallel group randomized clinical trial.** MD group final anthropometric measures (body weight, body mass index (BMI), WC, waist-hip ratio, and fat mass) decreased compared to baseline. MD group showed greater decrease in WC compared to CD group. Fasting glucose decreased in CD group. Both groups reduced glycated hemoglobin. Consumption of high-Ca diet from fat-free milk for 12 weeks was effective in reducing abdominal adiposity, but provided no additional effect on glycemic control in patients with T2DM. **CONCLUSION:** The increased consumption of fat-free milk (700 mg of additional Ca/d) seems to improve metabolic control of T2DM subjects.

RESUMO

GOMES, Júnia Maria Geraldo, D.Sc., Universidade Federal de Viçosa, dezembro de 2016. **Aumento do consumo de leite desnatado melhora a perda de peso, a composição corporal, os componentes da síndrome metabólica e as medidas de risco cardiometabólico em adultos com diabetes mellitus tipo 2.** Orientadora: Rita de Cássia Gonçalves Alfenas. Coorientadoras: Ana Paula Boroni Moreira e Hércia Stampini Duarte Martino.

INTRODUÇÃO: Sugere-se que o consumo de laticínios desnatados e de cálcio (Ca) dietético possa favorecer o controle glicêmico e do peso corporal, atuando na prevenção e tratamento do diabetes *mellitus* tipo 2 (DM2). Entretanto, não encontramos na literatura nenhum estudo em que se avaliou o efeito do aumento do consumo de leite desnatado no controle metabólico de individíduos com T2DM. **OBJETIVO:** Avaliar os efeitos do aumento da ingestão de leite desnatado no peso e composição corporal, nos componentes da síndrome metabólica e nas medidas de risco cardiometabólico em indivíduos portadores de DM2. **MÉTODOS:** Foram realizados dois ensaios clínicos randomizados com delineamento cruzado (N=14) (Artigos 1 e 2) e um com delineamento paralelo (N=36) (Article3). Indivíduos com DM2 e baixa ingestão habitual de Ca (< 600 mg/dia) participaram de duas fases com 12 semanas cada: rica em Ca proveniente de leite desnatado (MD) ou controle pobre em Ca (CD). Nos estudos cruzados (Artigos 1 e 2), as fases foram separadas por um intervalo de 8 semanas. Durante a intervenção, os participantes ingeriram diariamente em laboratório uma bebida contendo 700 mg de Ca proveniente de leite desnatado (MD) (equivalente a aproximadamente 3 porções de leite desnatado) ou 6.4 mg (CD) de Ca no desjejum. Além disso, foram prescritas dietas com restrição energética (-500 kcal/d) contendo 800 mg de Ca dietético/dia. **RESULTADOS: Artigo 1:** ***Fat-free milk enhances weight loss, improves body composition, and promotes glycemic control in adults with type 2 diabetes mellitus – a randomized clinical trial.*** O consumo de Ca foi equivalente a 1,200 mg/dia durante a fase MD e 525 mg/dia durante a fase CD. Observou-se maior redução do peso corporal, gordura corporal, perímetro da cintura (PC) e relação cintura-quadril após a fase MD. As concentrações séricas de 25-hidroxivitamina D e HOMA2-B% aumentaram, enquanto as concentrações séricas de ácido úrico, paratormônio e hemoglobina glicada reduziram após a

fase MD. Concluímos que o consumo de cerca de 3 porções de leite desnatado (700 mg de Ca adicional) exacerbou a perda de peso, melhorou a composição corporal e promoveu maior controle glicêmico em indivíduos diabéticos tipo 2 com baixo consumo habitual de Ca. **Artigo 2: Increased fat-free milk consumption, metabolic syndrome, and cardiometabolic outcomes in adults with type 2 diabetes *mellitus.*** Houve maior redução do PC, pressão arterial sistólica (PAS), pressão arterial diastólica (PAD) e índice do produto da acumulação lipídica (LAP) após a fase MD em relação à fase CD. Houve aumento da razão HDL (lipoproteína de alta densidade)/LDL (lipoproteína de baixa densidade) e redução do colesterol total, LDL-c, PAS, PAD e índice LAP apenas na fase MD. Concluímos que o consumo de cerca de 3 porções de leite desnatado (700 mg de Ca adicional) reduziu alguns componentes da síndrome metabólica e medidas cardiometabólicas em adultos diabéticos. **Artigo 3: *Dietary calcium from dairy, body composition and glycemic control in patients with type 2 diabetes: a parallel group randomized clinical trial.*** O peso corporal, índice de massa corporal (IMC), PC, relação cintura-quadril e gordura corporal reduziram no grupo MD comparados ao início do estudo. Houve maior redução do PC no grupo MD em relação ao grupo CD. A glicemia de jejum reduziu no grupo CD. Ambos os grupos apresentaram redução na hemoglobina glicada. O consumo de dieta hipocalórica rica em Ca durante 12 semanas foi efetivo na redução da adiposidade abdominal, mas não promoveu efeito adicional no controle glicêmico de pacientes com DM2. **CONCLUSÃO**: O aumento do consumo de leite desnatado (700 mg de Ca adicional por dia) parece contribuir o controle metabólico de portadores de DM2.

1. INTRODUCTION

Type 2 diabetes *mellitus* (T2DM) is a chronic metabolic disorder considered a public health problem due to its increasing incidence and prevalence all over the world (ADA, 2016). In 2013, the International Diabetes Federation reported that there were 382 million people with T2DM and projected that by 2035 the global tally of people with diabetes will be 592 million (Guariguata et al., 2014). The costs to treat the disease and prevent its complications are quite high for both the public health system and for patients and their families (ADA, 2016).

Several strategies have been studied to reduce the burden of T2DM aiming to improve the quality of life of individuals with T2DM. Weight loss positively correlates with glycemia reduction and improvement in the metabolic profile in impaired glucose tolerant (IGT), or diabetic's individuals (ADA, 2016). Moderate energy restriction (~ 500 kcal/d) associated with increased dietary calcium (Ca) or dairy consumption has been suggested to control insulin resistance (IR), metabolic syndrome (MetS), cardiovascular outcomes, and obesity in low habitual Ca consumers (Soares et al., 2011; Abargouei et al., 2012; Dugan and Fernandez, 2014; Visioli and Strata, 2014). In adults, the consumption of 1200 to 1500 mg of Ca/d seems to reduce lipogenesis and to increase lipolysis (Zemel et al., 2000; Candido et al., 2013), resulting in body fat, glycemia, and insulinemia reduction (Hussain et al., 2007; Nikooyeh et al., 2011; Ferreira et al., 2013). However, in Brazil, the mean daily dietary Ca intake is about 546.4 mg among men and 476.4 mg among women. Approximately 90% of the Brazilian adult population does not achieve the daily recommended dietary allowance (RDA) for Ca (IBGE, 2010; IOM, 2011).

The authors of systematic reviews and meta-analysis suggest that the beneficial effects of increased Ca intake are more significant in individuals who have a low habitual Ca intake (<600 mg/d) (Soares et al., 2011), who are prescribed a moderate energy restriction diet (-500kcal/d) (Abargouei et al., 2012), who use more bioavailable supplements such as dairy products (Freitas et al., 2012), or when dairy products are fat-free (Cândido et al., 2013).

Most of the studies about the effects of dairy components on body weight, body composition, and glycaemic control involve overweight subjects or athletes (Zemel et al., 2004; Bowen et al., 2005; Josse and Phillips, 2012; Torres et al,

2013), and only a few studies involve individuals with T2DM (Pittas et al., 2007; Pasin and Comerford, 2015). Dairy foods and dairy protein (mainly whey protein) consumption seem to improve insulin secretion in adults with T2DM (Pasin and Comerford, 2015). However, the different doses consumed, different dairy sources, and the short-term nature of the clinical trials do not allow effective dietary recommendations (Pasin and Comerford, 2015). In addition, some clinical trials involving individuals with T2DM used probiotic yogurt for the intervention group (Pasin and Comerford, 2015). It is well known that microbiota modulation also affect metabolism (Janssen and Kersten, 2015).

Possible mechanisms involving dairy components that may favor weight loss, cardiometabolic, and glycaemic control are still not well understood. Low-Ca diets increase calcitriol (1,25-dihydroxyvitamin D) and parathormone (PTH) concentrations, resulting in Ca^{2+} influx to adipocyte. Consequently, the activation of kinase proteins stimulates lipogenesis and suppresses lipolysis, increasing body fat and inducing insulin resistance (Zemel et al., 2000). Calcitriol also inhibits the expression of adipocyte uncoupling protein 2 (UCP2), which reduces mitochondrial fatty acid transport and fat oxidation (Zemel et al., 2000). Dietary Ca also interacts with dietary fatty acids in the gut, forming insoluble Ca fatty acid soaps, and therefore resulting in increased fecal fat excretion (Jacobsen et al, 2005). Milk proteins and their bioactive peptides are related to increased satiety, thermogenesis, and muscle mass preservation (Acheson et al., 2011; Devries and Phillips, 2015). Leucine seems to favor fat oxidation and muscle protein synthesis, avoiding lean mass loss (Sun and Zemel, 2007). This branched-chain amino acid (BCAA) has also been associated with improved insulin sensitivity, reduced oxidative stress and reduced blood pressure (BP) (Visioli and Strata, 2014; Hirahatake et al., 2014). Milk minerals, such as Ca, zinc (Zn) and magnesium (Mg) play a central role in the action of insulin and in BP control (Visioli and Strata, 2014; Hirahatake et al., 2014). Ca is essential to convert proinsulin to insulin and to promote insulin release (Gilon et al., 2014). Ca also favors vasodilatation, reducing BP (Dugan and Fernandez, 2014). Zinc is involved in the synthesis, storage and secretion of insulin, as well as in maintaining the conformational integrity of insulin. Magnesium is an essential ion that helps in the secretion of insulin and also acts as a cofactor of several enzymes in the metabolism of carbohydrates (Yahya et al., 2011). Vitamin D may reduce cardiovascular risk by maintening

Ca homeostasis, stimulation of insulin production and release, and regulation of the renin-angiotensin-aldosterone system, which leads to improved BP control (Rice et al., 2013). The beneficial effects of Ca/ dairy on obesity, MetS, and T2DM control may also be associated with intestinal microbiota and endotoxemia modulation, as discussed in the “Literature Review” (Gomes et al., 2015; Gomes et al., 2017), since some studies have demonstrated the importance of these intestinal parameters in the development of chronic diseases (Cani et al., 2008; Cani et al., 2009; Larsen et al., 2010; Wu et al., 2010).

Considering the few studies involving T2DM subjects and the possible effects of dairy on weight loss, body composition, glycemic control, and cardiometabolic outcomes, controlled intervention studies are necessary to evaluate the effects and mechanisms of the action of increased fat-free milk consumption on metabolic changes related to T2DM, aiming to improve the metabolic profile in these patients.

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2. LITERATURE REVIEW

2.1 REVIEW ARTICLE 1 - Published in *British Journal of Nutrition* (2015), **114**, 1756–1765. DOI: 10.1017/S0007114515003608

Title:

Could the beneficial effects of dietary calcium on obesity and diabetes control be mediated by changes on intestinal microbiota and integrity?

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Short-title: Calcium, microbiota and gut integrity

Key words: Calcium: Gut microbiota: Intestinal permeability: Endotoxin: Lipopolysaccharide

Abstract

Evidence from animal and human studies has associated gut microbiota, increased translocation of lipopolysaccharide (LPS) and reduced intestinal integrity (II) with the inflammatory state that occurs in obesity and type 2 diabetes mellitus (T2DM). Consumption of Ca may favour body weight reduction and glycaemic control, but its influence on II and gut microbiota is not well understood. Considering the impact of metabolic diseases on public health and the role of Ca on the pathophysiology of these diseases, this review critically discusses possible mechanisms by which high-Ca diets could affect gut microbiota and II. Published studies from 1993 to 2015 about this topic were searched and selected from Medline/PubMed, Scielo and Lilacs databases. High-Ca diets seem to favour the growth of lactobacilli, maintain II (especially in the colon), reduce translocation of LPS and regulate tight-junction gene expression. We conclude that dietary Ca might interfere with gut microbiota and II modulations and it can partly explain the effect of Ca on obesity and T2DM control. However, further research is required to define the supplementation period, the dose and the type of Ca supplement (milk or salt) required for more effective results. As Ca interacts with other components of the diet, these interactions must also be considered in future studies. We believe that more complex mechanisms involving extraintestinal disorders (hormones, cytokines and other biomarkers) also need to be studied.

Introduction

Calcium (Ca) is the major mineral component of the skeletal system and is also an essential nutrient required for blood clotting, nerve conduction, muscle contraction, besides being essential for endocrine and hormone secretion[1]. In adults, adequate Ca intake as recommended in the Dietary Reference Intakes (DRIs) seems to prevent obesity[2] and type 2 diabetes *mellitus* (T2DM)[3]. Possible mechanisms involving Ca that may favor weight and glycemic control are still not well understood. The results of *in vitro* and animal studies suggest that low-Ca diets increase calcitriol (1,25-dihydroxyvitamin D) and parathormone concentrations, resulting in Ca influx into adipocytes. Increased intracellular Ca^{2+} activates lipogenesis (mediated by fatty acid synthase) and suppresses lipolysis (hormone-sensitive lipase), increasing body fat and inducing insulin resistance (IR)[4]. Calcitriol also inhibits the expression of adipocyte uncoupling protein 2 (UCP2), reducing mitochondrial fatty acid transport and lipid oxidation [4]. Another possible mechanism is the interaction between dietary Ca and fatty acids in the gut, forming insoluble Ca fatty acid soaps, which in turn increases fecal fat excretion and reduces dietary energy[5].

Influx of Ca^{2+} into muscle cells promotes GLUT4 translocation and, hence, increases glucose uptake and insulin sensitivity in skeletal muscle[6]. A moderate influx of Ca into pancreatic beta-cells is essential for converting proinsulin to insulin and promoting insulin release[7]. In healthy adults, Ca supplementation increased the concentrations of gastrointestinal insulinotropic hormones, especially glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1)[8], and, hence, increased insulin sensitivity indirectly.

The beneficial effects of calcium ingestion could be associated with intestinal microbiota modulation and with increased integrity of the intestinal mucosa since the results of human and animals' studies indicate the importance of these intestinal parameters on obesity and diabetes development[9-14]. Gut microbiota constitutes an important factor that affects nutrient absorption, energy homeostasis, body weight control, and IR[13]. Intestinal permeability can be defined as the property that allows solute and fluid exchange between the intestinal lumen and the tissues[15]. Gut barrier is a functional unit that prevents this exchange, composed of gut microbiota, mucus, polarized epithelial cell

membrane, tight junctions, and the innate and adaptive immune cells forming the gut-associated lymphoid tissue[15-18]. Integrity of these barrier structures is essential to maintain normal intestinal permeability[18]. When the intestinal barrier is unimpaired, permeability is highly selective, avoiding the entrance of undesirable solutes, pathogenic microorganisms, and toxins to the blood stream [16-18]. Integrity breakdown and increased intestinal permeability have been associated with obesity and diabetes aetiopathogenic mechanisms through the activation of proinflammatory pathways[9, 12, 19].

In animal studies, increased intestinal permeability caused metabolic endotoxemia (measured by the translocation of bacterial lipopolysaccharide (LPS) derived from Gram-negative intestinal microbiota into the peripheral circulation), low-grade inflammation, and glucose intolerance[9, 20]. Studies have shown differences on gut microbiota composition and higher concentrations of circulating endotoxins when obese and/or diabetic subjects are compared with lean and normoglicemic ones [16, 21, 22, 23].

Considering the low-Ca consumption by industrialized populations[24] and the increase in the worldwide prevalence of obesity and T2DM, this review aims to discuss the influence of dietary Ca on gut microbiota composition and intestinal integrity in order to elucidate a possible therapeutic strategy for the prevention and/or treatment of obesity and T2DM.

Methods

Medline/PubMed, Scielo and Lilacs were searched using thefollowing terms: calcium, dairy food, gut or intestinal or gastrointestinal microbiota, gut or intestinal or gastrointestinal barrier, gut or intestinal or gastrointestinal permeability, lipopolysaccharide, endotoxins, metabolic endotoxemia, tight junction. For data searches, the terms in English, Spanish or Portuguese were used either alone or in association. Review and original studies published from 1993 to 2015 were selected according to their titles and abstracts. In vitro studies were excluded. Each selected manuscript was critically analysed.

Gut microbiota and intestinal integrity: How might dietary Ca work?

Human gut microbiome may be affected by short-term (about a few days) dietary Ca intake [25]. Dietary Ca may affect gut microbiota and intestinal integrity through mechanisms involving gastric acid secretion, bile acid (BA) and

fatty acid (FA) precipitation, competition among intestinal bacterial communities, and changes on fermentation products in the colon and on intestinal mucosal integrity (**Figure 1**). **Table 1** (Supplementary Material) briefly describes studies that evaluated the effects of Ca supplementation on intestinal integrity in animals and humans.

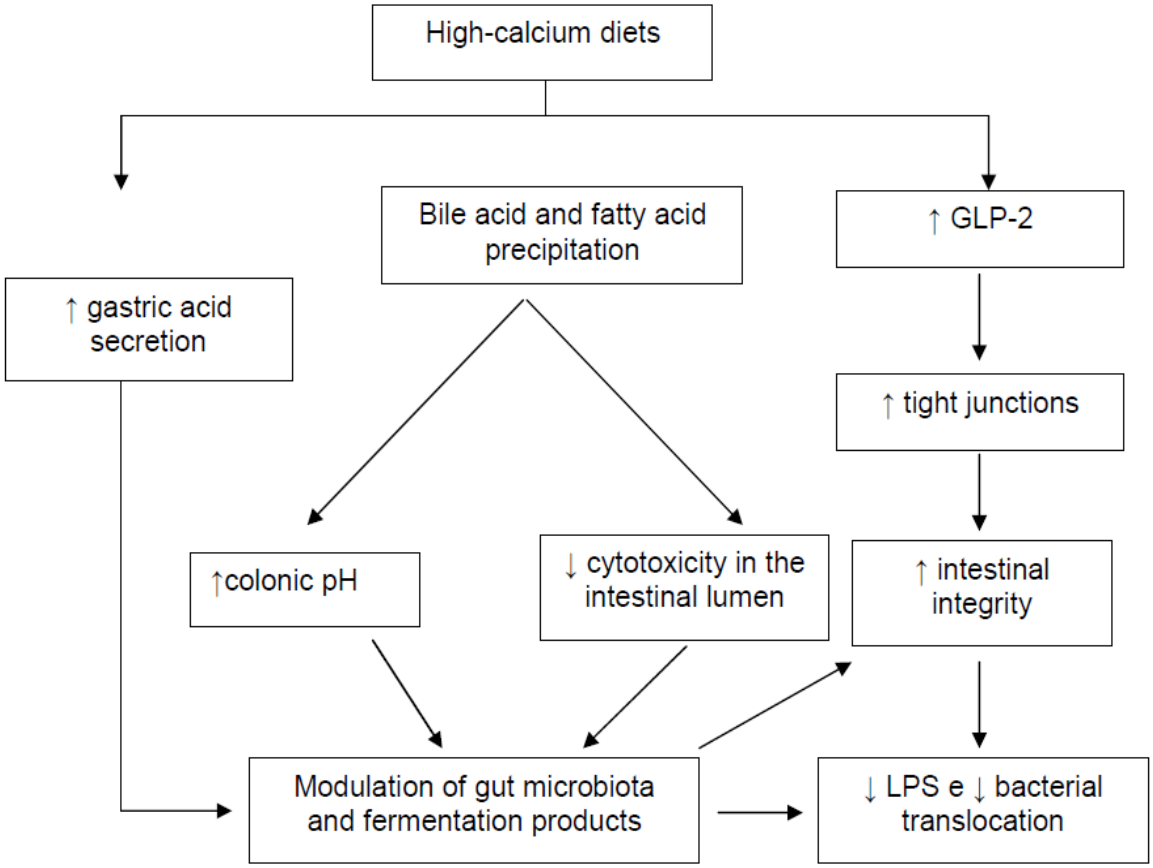

Figure 1. Possible mechanisms explaining the effects of high-calcium diets on intestinal integrity and gut microbiota. High-calcium diets seem to change the intestinal environment through the following mechanisms: (1) increasing gastric secretion leading to increased gastric pH and reduced number of viable bacteria; (2) causing bile acid and fatty acid precipitation, increasing colonic pH and reducing cytotoxic components (especially NEFA and ionised secondary bile acids) that damage the epithelial cells; and (3) increasing glucagon-like peptide-2 (GLP-2) secretion, which has a trophic effect on intestinal mucosa and reduces gene expression of tight junctions (occludin and zonula occludens-1). These mechanisms may reduce bacterial and lipopolysaccharide (LPS) translocation, by bacterial fermentation and intestinal microbiota modulation, leading to a highly selective and controlled intestinal permeability.

1) Antimicrobial effect of gastric acid secretion

High-Ca diets (30 mmolCa/l [26] and 180 mmol Ca/kg diet [27]) are associated with reduced number of viable bacteria in the stomach. A high intraluminal concentration of Ca^{2+} stimulates the release of gastrin and, consequently, increases acid secretion[28]. Several bacterial species (e.g. Salmonella) are destroyed by gastric acid[29]. However, some factors, such as the buffering effect of food, gastric emptying rate, and mechanisms of bacterial resistance, interfere with the interaction of gastric acid and ingested bacteria[30]. Wistar rats (n = 9 per group) were fed *ad libitum* for 12 days on a low-Ca diet (control: lactose-free low-Ca milk, 3.8% fat, 6 mmol Ca/l), regular lactose-free milk (3.7% fat, 30 mmol Ca/l), acidified milk, or yoghurt (both prepared with regular lactose-free milk)[26]. These rats were orally infected with *Salmonella enteritidis* just after food consumption. Animals fed on yoghurt had lower fecal excretion of bacteria than those in the other groups. The authors suggest that the lower gastric emptying rate after yoghurt consumption could have prolonged the exposure to gastric acid and, thus, reduced the effectiveness of the inoculation. Thereafter, fecal Salmonella excretion declined rapidly in all high Ca-groups compared with the control group[26]. Another study conducted by the same authors showed similar results[27]. Mice infected with *S. enteritidis* were fed high-, medium-, or low-Ca diets (n = 8 per group; Ca in the form of CaHPO4: 180, 60, and 20 mmol/kg diet respectively). Compared with the low-Ca diet, the medium- and high-Ca diets favored bacterial colonization[27]. High-Ca intake (30 mmolCa/l [26] and 180 mmol Ca/kg diet [27]) probably increased the gastric acidity and, hence, reduced viable counts of *S. enteritidis*[26, 27]. Other studies also showed the antimicrobial effect of gastric acid[29, 31], which potentially modified the endogenous microbiota by reducing viable bacteria in the gut. In general, few microorganisms, such as *Helicobacter pylori*, some streptococci, lactobacilli, and probiotics can survive extremely acidic conditions within the stomach[32, 33]. However, in some studies [26, 27], gastrin release and/or acid secretion were not measured. Therefore, we cannot conclude that the results of those studies[26, 27] were mediated by changes in gastric secretion. High-protein content and the liquid state of the diets facilitated bacterial survival within the stomach[29]. Furthermore, some dairy products components, such as immunoglobulins, peptides, lactoferrin, lactoperoxidase, and lysozyme, have antimicrobial effects. Other dairy components, such as lactose, peptides, and

probiotics stimulate potentially beneficial bacteria that compete with pathogens for nutrients and attachment, and enhance the mucosal immune response to pathogens[34, 35].

2) Bile acid and fatty acid precipitation: Reducing luminal cytotoxicity

Due to the low pH, dietary Ca in the stomach exists in dissociated form, while, in the small intestine, there is an equilibrium between its dissociated and non-dissociated forms. In the distal ileum and the colon, where pH is higher than 6, Ca interacts with dietary phosphate, forming an insoluble complex that precipitates intestinal BAs and FAs. Hence, Ca increases their fecal excretion in animals[27, 36-38] and humans[39-44]. BA precipitation increases the *de novo* synthesis of BAs from cholesterol in the liver and, hence, reduces serum cholesterol. FA precipitation reduces fat absorption, increasing fecal energy loss[5].

Primary BAs (cholic acid and chenodeoxycholic acid) are synthesized in the liver from cholesterol and then conjugated with either glycine or taurine, often called bile salts. About 5% of BAs are deconjugated and dehydroxylated by bacterial enzymes in the intestine to form secondary BAs (deoxycholic acid and lithocholic acid), which are more cytotoxic. The dehydroxylation process involves the removal of the OH group at the 7-position of the steroid nucleus (also termed 7-dehydroxylation). Deconjugation results in amino acid side chain cleavage. Among intestinal bacteria, 7-dehydroxylase was detected in the *Eubacterium* and *Clostridium* genera but not in lactobacilli and bifidobacteria [45, 46]. BA hydrolysis is mediated by several gut microbiota genera, including *Clostridium*, *Bacteroides*, *Lactobacillus*, *Bifidobacterium* and *Enterococcus*[45]. Approximately 95% of BAs are reabsorbed in the distal ileum and return to the liver (enterohepatic circulation of BAs). About 400-800 mg BAs/day elude enterohepatic circulation and are subjected to extensive modifications by the endogenous colonic microbiota. Secondary BAs formed by colonic bacteria can be absorbed passively and, thus, may contribute to the BA pool[28].

Small amount of non-esterified free FAs (NEFAs) and ionized secondary BAs that reaches the colon can damage the intestinal epithelium and, thus, increase colonic permeability[47]. Therefore, BA and FA precipitation caused by dietary Ca promotes cytoprotective effects by reducing the bacteria's formation

of cytotoxic surfactants. BA and FA precipitation ultimately maintain the integrity of colonic epithelium.

Measuring the BA and FA concentrations in the soluble portion of the feces (so-called fecal water (FW)) is more reflective of luminal cytotoxicity than measuring the total fecal BA and FA concentration [48]. FW refers to the supernatant obtained after feces intense centrifugation. FW contains aqueous soluble BAs and FAs not linked to other fecal compounds[49]. In some studies, a high-Ca diet reduced the BA and FA concentration in FW[26, 27, 36-38, 40, 41]. A high-Ca diet also reduced FW cytotoxicity by precipitating cytotoxic surfactants, resulting in lower colonic epithelium damage and higher resistance to infections[26, 27, 36-38, 40, 41, 47, 50-52]. High-Ca diets contained 30 mmol/l [26], 225 µmol/g diet [36, 40], 120 [47,51], 150 [37], or 180 [27, 38] mmol/kg diet, and 4.8g/kg diet [52] in the rats studies, and 1200 mg/day in the human study [41]. Some effects of cytotoxic surfactants are cell membrane disruption, inflammatory reactions activation, and epithelium hyperproliferation enhancement [24, 32, 53]. Guarner[54] criticized the use of erythrocytes instead of intestinal epithelial cells to analyze FW cytotoxicity, used in these studies [26, 27, 36-38, 40, 41, 47, 50-52]. Red blood cells are susceptible to changes in pH resulting from the production of short chain fatty acids (SCFAs) after the fermentation of non-digestible carbohydrates. So, hemolysis caused by changes in pH by SCFA may not reflect epitheliolysis [54]. On the other hand, intestinal cells normally use organic acids as an energy source[54]. However, the erythrocyte assay has previously been validated[49]. In a mice study, the type of dietary fat influenced fecal FA excretion. Ca-PUFA soaps were more soluble and, therefore, better absorbed than Ca-saturated FA soaps[36]. The decreased absorption of intestinal fat (mainly saturated fat) caused by dietary Ca is of interest for the improvement of obesity control. By contrast, FA precipitation was independent of the source of Ca (milk, Ca-carbonate or Ca-phosphate (CaP))[37] and dietary phosphate content (75, 125 or 275 mmol/g diet)[40].

Increased fecal fat excretion after Ca supplementation seems to favor weight control. An average daily intake of 1200 mg Ca results in the excretion of 5.2g fat/day and a weight loss of 2.2 kg/year[55]. Therefore, it is possible that this mechanism contributes to obesity control but does not fully explain the effect of Ca on weight loss[5]. We believe that the impact of dietary Ca on weight loss is also related to dysbiosis attenuation. In this context, a high-Ca diet (>1100

mg/day) seems to modulate gut microbiota by reducing the number of BAs and FAs available for bacterial metabolism. It is possible that this effect increases *Lactobacillus* and reduces bile tolerant bacteria, as discussed below.

3) Resistance to pathogens and changes in gut microbiota composition

In rodents, high CaP diets increased fecal lactobacilli excretion[38, 50, 52], reduced fecal enterobacteriaceae excretion[50, 56], and increased resistance to *S. enteritidis* after 6-7 days of infection[38,50, 52, 56]. Calcium concentration of these diets were previously mentioned [38, 52], except the concentration adopted in the study conducted by Ten Bruggencate et al. [50, 56], which was 100 mmol Ca/kg diet. Rats were fed on diets with 60 g/kg of cellulose (control), fructo-oligosaccharides (FOS), or inulin with either a low (30 mmol/kg) or a high (100 mmol/kg) Ca concentration. After two weeks of adaptation, the animals were infected with *S. enteritidis*. During the following six days, FW cytotoxicity increased in the rats on inulin and FOS diets, but the high-Ca diet minimized this adverse effect[50]. In another study, rats infected with *S. enteritidis* were fed on different sources of Ca supplements (CaP, milk Ca, calcium chloride, or calcium carbonate (a total of 100 mmol Ca supplement/kg)) or a low-Ca diet (20 mmol CaP/kg). After an adaptation period of two weeks, all the Ca supplements reduced infection and increased resistance to *Salmonella*[56]. Effects of Ca salts were similar to milk Ca[56], suggesting a strategy to increase Ca intake in cases of restricted consumption of dairy foods, e.g. lactose intolerance.

The authors suggest that the resistance to pathogens was due to BA and FA precipitation and, consequently, to reduced cytotoxic surfactants in FW after CaP supplementation[38, 50, 52, 56]. However, only Bovee-Oudenhaven et al.[38] evaluated BA and FA fecal excretion. Gram-negative bacteria (such as *Escherichia coli* and *Salmonella spp.*) are more bile-tolerant than Gram-positive bacteria (such as some species of *Bifidobacteria*, *Bacillus*, *Lactobacillus* and *Enterococcus*)[45]. Therefore, the reduction in cytotoxicity in the intestinal lumen (reduction in the lytic activity of luminal surfactants), induced by dietary CaP could improve the growth of *Lactobacillus* and other Gram-positive bacteria compared to that of Gram-negative bacteria.

Another mechanism by which dietary CaP interferes with resistance to pathogens is by binding directly to *Salmonella* and, hence, increasing the excretion of fecal bacteria, which is also known as bacterial shedding. This

reduces pathogen competition and, so, enhances the growth of lactobacilli. *In vitro*[38, 56] but not *vivo*[52] studies confirmed this mechanism. In an *in vivo* study[52], rats (n = 8 per group) infected with *Salmonella* were treated with an antibiotic and were either fed on the control (1.2 g/kg diet) or on a high-Ca diet (4.8 g/kg diet). Both diets did not decrease *Salmonella* colonization (measured by the excretion of fecal bacteria). The authors rejected the hypothesis that the binding of the CaP complex to *Salmonella* has a significant effect *in vivo* and, therefore, suggested that surfactant precipitation may increase endogenous microbiota.

Research on pigs showed different effects of CaP in intestinal lactobacilli colonization. In general, high CaP intake did not affect *Lactobacillus spp.* growth [57-59] in the pigs' stomachs, ilea, or colons. Only in the study by Mann et al.[60] did high- versus adequate-CaP diets increase *Lactobacillus spp.* growth in the stomach. These studies were performed with growing[57] or weaned [58-60] pigs (n = 8 per group) fed on high CaP diets: with an ileal pectin infusion[57], with a high or low β-glucan content[58], associated with a corn diet, or associated with a wheat-barley diet[59, 60]. High CaP diets contained 15 g Ca/kg diet [50], 10g Ca/kg diet [51], and 14.8 g Ca/kg diet [59, 60]. The main difference between these pig studies that explain the changes in gastrointestinal microbiota was the methodology used to quantify bacterial communities. Studies in which no effects on lactobacilli count were observed used quantitative PCR[57-59], while Mann et al.[60] used the pyrosequencing of 16S rRNA genes. Because of the diversity and complexity of bacterial communities between species, the latter technique has been recommended. It has the capacity to sequence multiple fragments simultaneously and, thus, achieves more rapid and accurate bacterial genome sequences[59, 61]. Although some authors criticize pyrosequencing for the phylogenetic classification of sequences obtained at the species level[61]), Mann et al.[60] did not use it for pyrosequencing the total genome. Rather, they only used it for the 16S rRNA gene. Thus, they demonstrated increases on operational taxonomic units (OTUs) for *Lactobacillus*, indicating an increase in the number of microorganisms of this genus. This is extremely beneficial due to the effects of these bacteria on intestinal health.

CaP diet modulated gastrointestinal microbiota in all pig studies, but the results were very different. Due to that, these studies will not be described in detail (see Table 1, Supplementary Material). In general, CaP intake over 14

days increased *Clostridium* cluster XI and XVIa in the ilea, ceca, and colons of weaned pigs[57-60]. Several species of *Clostridium* cluster IV and XIVa produce butyrate, which is an important energy source for colonocytes[62].

A double-blind, placebo controlled, crossover study evaluated the composition of gut microbiota after CaP and probiotic supplementation in humans [43]. Participants (32 healthy men and women aged 25±5 y and BMI of 22±3 kg/m^2) consumed a probiotic drink containing 10^{10} CFU/d *Lactobacillus paracasei* alone or in combination with bread containing CaP (1 g/day) for four weeks. CaP supplementation decreased total cholesterol, LDL-C, and the LDL/HDL ratio, and increased fecal pH and the fecal excretion of secondary BAs compared with supplementation with probiotics or placebo alone. Probiotic supplementation, alone or with CaP, increased fecal *Lactobacillus* excretion. The authors explained these effects as resulting from BA precipitation by amorphous CaP, particularly when BAs are deconjugated by probiotics, and from the increased fecal excretion of these components. A less cytotoxic intestinal lumen, which contains low concentration of cytotoxic surfactants, might favor the growth of lactobacilli and reduce blood cholesterol concentration due to the increased conversion of cholesterol into BAs[43].

The results of the studies with mice, pigs, and humans are difficult to compare because of the diversity of microorganism species in different hosts. Within the same species (mouse, pig, or man), the intestinal maturation stage can also influence gut microbiota composition. For instance, *Enterococcus spp.* in the ileum were observed to decrease and increase in growing and weaned pigs respectively after CaP supplementation[57, 59]. In humans, gut microbiota is dominated by bifidobacteria in the first 2-3 years of life (especially among breastfed children), remaining relatively stable in adults with 90% of the bacteria from the *Bacteroidetes* and *Firmicutes* phyla. In the elderly, gut microbiota becomes less diverse again (higher *Bacteroides* to *Firmicutes* ratio, an increase in *Proteobacteria* and a decrease in *Bifidobacterium*)[63]. Human gut microbiota also varies according to genetic background, diet, antibiotic use, and the health status of the host[64]. Therefore, the interaction between Ca and other dietary nutrients (such as lactose, dietary fiber, and probiotics) probably influences its effect on microbiota composition and activity.

Furthermore, the different results observed may be due to the variety of procedures employed on microbiological analyses. These range from simple

techniques such as counting colony forming units in mouse studies[38, 50, 52] to sophisticated molecular biology techniques such as qPCR and pyrosequencing 16S rRNA genes in pig and human studies[43, 57-60]. The variety in materials or parts of the gastrointestinal tract used to evaluate microbiota composition may also make comparisons difficult. Mice and human studies used feces[38, 43, 50, 52], while pig studies used different viscera (stomachs, small intestines, ceca, and colons)[57-60].

Differences between BAs in mice (predominantly taurine-conjugated) and those in pigs (glycine-conjugated) also partly explain the diversity in results. In general, unconjugated BAs and glycine-conjugated BAs are more strongly precipitated by CaP than taurine-conjugated BAs are [38]. Taurine-conjugated to glycine-conjugated BA ratio in human bile is usually 3:1, which is more similar to that in pigs than to that in rodents[45].

Obesity is associated with changes in gut microbiota composition in animal and human research[10, 65]. Some studies show an increase in the *Firmicutes* to *Bacteroidetes* ratio[66], and others only show an overall decrease in *Bacteroidetes* and no change in *Firmicutes*[22]. There are also differences between of obese and lean subjects gut microbiota [65]. However, it is not yet clear whether obesity leads to dysbiosis or vice versa. If the effects of dietary Ca on lactobacilli growth are confirmed in human clinical trials, Ca supplementation will be an useful strategy in obesity treatment.

4) Change in fecal pH and in fermentation products

Most mouse[26, 50, 67] and human[- 41, 43] studies have found an increase in fecal pH after high-Ca diets (30 mmol Ca/l [26], 100 [50], 120 [67] mmol Ca/kg diet in mouse studies, and, 1000 [43] or 1200 Ca mg/day [41] in human studies). Other mouse studies[36], as well as pig studies[58, 59], did not reveal such differences, and only one study showed a decrease in fecal pH as a result of Ca supplementation[37] (225 µmol Ca/g diet [36], 10 [58] or 14.8 [59]gCa/kg diet, and 1g/day[37]). The decrease in fecal pH, caused by products of the colonic fermentation of non-digestible carbohydrates, supports the growth of beneficial bacteria (particularly bifidobacteria and lactobacilli)[68].As previously mentioned, dietary Ca is soluble in acids, and it precipitates at alkaline pH. Gastric acidity (pH 1 to 3) is sufficient to release Ca complexed to salts or foods. Thus, the ionized Ca can be absorbed via transcellular active transport in the duodenum

and proximal jejunum and via a passive paracellular process throughout the ileum. Less than 10% of Ca absorption occurs in the colon, and it involves the paracellular and transcellular pathways[69]. About 25% to 35% of ingested Ca is generally absorbed. As pH increases from ileum to colon, the intestinal phosphate concentration also increases, causing Ca precipitation and Ca absorption reduction [28]. Therefore, an increase in Ca intake may increase the buffering capacity of feces due to the formation of an amorphous CaP complex. In the studies discussed in our review, the acidification caused by bacterial fermentation may have been buffered by the high amounts of CaP in the colonic lumen (quantities described in the first paragraph of this session), causing no change or increase in fecal pH[26, 36, 41, 43, 50, 58, 67]. The buffering effect is suggested by the increase in the fecal excretion of Ca and phosphate[36, 37, 41, 43] and/or the decrease in BA and FA concentration in FW[26, 36, 37, 41, 43].

In the animal research that evaluated the effect of Ca supplementation on the products of bacterial fermentation, there was an increase in acetate in the ilea[57] and caproate in the colons[59] of pigs. There was also an increase in lactate in the ceca[47], and in propionate and butyrate in the feces of mice[70]. Metzler-Zebeli et al.[58] observed a decrease in propionate in the ceca of pigs. The sources and quantities of fiber used to stimulate colonic fermentation varied among the studies (60 g pectin/day[57]; 60 g FOS/kg diet[47]; 36 g crude fiber/kg diet[59]; 6% (w/w) GOS[70]). The use of probiotics in humans[71] and animals[72] increases Ca absorption, especially in the colon. However, even with this increase, it is possible that much of the ingested Ca transits through the colon without being absorbed[71].

In a crossover study, isoenergetic diets with similar macronutrient compositions, rich in semi-skimmed milk (1.7 g Ca/day) or cow's semihard cheese (1.7g Ca/day), and ingested by 15 healthy adult men over 14 days increased fecal SCFAs (acetate, propionate, and butyrate) in comparison to the control diet (which was rich in butter with ~ 360 mg Ca/day)[44]. In comparison to the control diet, both experimental diets increased fecal fat excretion, mainly in the milk group. Cheese intake resulted in higher fecal concentrations of propionate and butyrate while milk intake promoted greater fecal excretion of acetate. These results indicate that milk and cheese stimulate bacterial activity differently. According to the authors, further studies are needed to explore the reasons for this difference. A possible interference factor is the protein profile of

cheese (mainly casein, absorbed slowly) and of milk (20% casein and 80% whey protein, with the latter absorbed faster)[44].

Colonic fermentation of fiber mainly generates lactate and SCFAs. The amount and type of these metabolites formed depends on gut microbiota composition, the substrate, and the intestinal transit time[73]. Acetate is produced by several groups of bacteria and comprises about 60% to 75% of total SCFAs. In addition, acetate is metabolized in peripheral tissues (through the formation of acetyl-CoA) and/or used for butyrate synthesis [73]. The number of microorganisms that can produce propionate and butyrate is low. Propionate is mainly produced by *Bacteroides spp., Clostridium* cluster IX*, Mitsuokella,* and *Veillonella spp.*[58]. Proprionate is metabolized in the liver via glycogenesis and is a lipolysis inhibitor and an inhibitor of the formation of acetyl-CoA from acetate[44]. Butyrate, an inhibitor of acetate synthesis, is the main energy source for colonocytes, followed by acetate then propionate[44, 74]. Some SCFAs production, such as butyrate, is important not only as an energy source for colonocytes, but it also prevents the accumulation of potentially toxic metabolites such as D-lactate. Also, butyrate acts in visceral sensitivity and intestinal motility, regulates transcellular fluid transport, reinforces the gut barrier, and reduces mucosal inflammation and oxidative stress[75]. *Eubacterium rectale, Clostridium coccoides,* and *Roseburia*, which belong to the genus *Clostridium* cluster XIVa, are the largest butyrate producers[69]. Some species of *Clostridium* cluster XIVa can convert lactate to butyrate, while some cluster IX members can convert lactate to propionate[76].

Lactic acid is a primary metabolite of fermentation in the cecum[73]. The production of lactic acid and SCFAs lowers the pH, inhibiting the activity of microorganisms that metabolize lactate, for example, propionate-producing bacteria[77]. Excessive lactate production culminates in its accumulation in the colon since it has low intestinal absorption[76.77]. Overduin et al.[70] suggest that dietary CaP supplementation influences this fermentation since the amorphous complex formed acts as a buffer against cecal acidification by lactate, thereby accelerating lactate conversion to SCFAs in the cecum. According to the authors, colonocytes' rapid uptake of butyrate may have masked SCFA production, which explains why they observed lower concentrations of butyrate in the colons of rats that had been fed prebiotics. Moreover, since lactate is less absorbable, it can accumulate in the colon in larger quantities[70].

Using the quantification of fermentation products to evaluate bacterial metabolic activity may be biased. Many of these products can act as intermediate substrates (i.e., lactate and acetate) and, therefore, may be associated with the metabolic activity of bacterial producers and/or bacterial users of these substrates. For example, an increase in lactate concentrations may indicate an increase in lactate producers or a decrease in lactate users. Therefore, it does not allow definitive conclusions. Moreover, about 95% of the SCFAs produced by bacterial fermentation are absorbed by colonocytes during the intestinal transit. Therefore, a lack in the alteration of these components may not represent real changes in the gut microbiota. Perhaps it represents the more effective use of the components, mediated by diet[70, 76, 77].

Obese subjects and animals have more SCFAs in their ceca than lean ones. This seems to favor higher energy storage after the intake of non-digestible carbohydrate[66, 78] and lower intestinal transit time induced by the hormone peptide YY[79]. Overall, this favors weight gain. Although the effects of Ca have not been confirmed, considering the results of the studies analyzed, it is possible that the increased fecal fat excretion and the modulation of gut microbiota that resulted from high-Ca diets (~1100 mg Ca/ day in the human studies [41, 43]) counteracted these effects.

5) Effects on intestinal permeability and integrity

Paracellular permeability allows substance movement between adjacent cells, by a passive process. In contrast, through transcellular permeability transport can occur across the cells, and it involves both active and passive processes [17]. Several high-Ca salts (CaP, milk, Ca carbonate and Ca chloride) decreased intestinal permeability in rats (100 [56] or 120 mmolCa/kg [47, 51, 67, 80], and 1.5% Ca [81]). Most of these studies used oral administration of EDTA chromium (Cr-EDTA) as a marker of intestinal paracellular permeability[47, 51, 67, 80, 81]. As Cr-EDTA is stable throughout the gastrointestinal tract, its excretion in the urine reflects total intestinal permeability[67]. Sugars, such as lactulose (reflecting paracellular permeability) and mannitol (transcellular permeability) are usually ingested to measure region specific permeability. These sugars are readily degraded by colonic microbiota. Thus, the urinary excretion rate of these sugars (lactulose to mannitol ratio) is used to express the small intestine permeability[82].

High-Ca diets (quantities described in the previous paragraph) decreased Cr-EDTA urine excretion, and the increased lactulose to mannitol ratio in rats compared with that associated with the control diet[67]. However, there was no statistical difference when the urinary excretion of the lactulose and that of mannitol were analyzed individually. Based on individual lactulose results, the authors concluded that dietary Ca did not affect the permeability of the small intestine. Consequently, they questioned the relevance of the lactulose to mannitol ratio and recommended mensuring the excretion of each sugar individually[67]. This is relevant because, in some situations where both sugars are excreted in large or small quantities, the ratio remains unchanged[17]. Obese women appear to have higher urinary excretion of lactulose and mannitol, while their lactulose to mannitol ratio does not vary from that of lean women[83]. The use of large probes as lactulose is the best to analyze macromolecules passage through the intestinal barrier, such as dietary antigens and other components derived from bacteria[17]. Another relevant example involves celiac patients, who tend to have low mannitol excretion due to villous atrophy, while their lactulose excretion is high. When calculating the lactulose to mannitol ratio, this information does not lead to accurate results[84]. Therefore, it is suggested that Ca supplementation mainly affects colonic permeability[67], which is expected, considering the previous discussion about the effects of Ca on BA and FA precipitation in the colon. This effect reduces cell damage and, consequently, increases the integrity of the epithelial mucosa.

In a transgenic animal model of colitis induction, Ca supplementation prevented colitis-induced increase in the expression of extracellular matrix remodeling genes such as matrix metalloproteinases, procollagens, and fibronectin. This suggests that Ca strengthened the integrity of the colonic mucosa[51]. Even in animal models, high dietary Ca (90 mmol/kg) prevented the FOS-induced increase in intestinal permeability (measured by Cr-EDTA) only when phosphate content was medium (70 mmol/kg diet) or high (160 mmol/kg diet). This was not the case with low-phosphate diets (5 mmol/kg diet). The effect was attributed to the buffering capacity of the colonic lumen due to the formation of a CaP complex, which could reduce luminal cytotoxicity. In this respect, the phosphate content of the diet is not decisive, but it is necessary for the Ca's effect on intestinal permeability[47]. Therefore, based on Schepens et al.[47], a ratio of about 1:1.3 of Ca/P can affect colon permeability.

Extracellular Ca (luminal Ca) was essential for intestinal tight junctions maintenance [85]. Tight junctions are apical intercellular joints, which contain transmembrane proteins, cytoskeleton components, and cytoplasmic plaques[86]. These junctions act on cellular adhesion, intracellular signaling, protection against extracellular entrance, and paracellular transportation of substances to the intestinal lumen[87]. Among the various tight junction proteins, the transmembranes proteins (such as occludin and claudin) and cytoplasmic plaques (como a zonula occludens) are important for paracellular transport[88]. Low-Ca and/or low-vitamin D diets reduce tight junction gene expression in calbindin-null mice[88], suggesting the importance of this mineral for the synthesis of tight junction proteins and, therefore, for paracellular permeability. The intact microbiome appears to be essential for normal gut-brain axis signaling and the expression of calbindin, restoring the intrinsic and extrinsic enteric nerve function in germ-free mice, and causing changes to intracellular calbindin concentrations[89]. Thus, it is believed that the microbiome may contribute to improve dietary Ca absorption.

CaP supplementation (1g/ day) during 3 weeks increased glucagon-like peptide- 1 (GLP-1) and glucagon-like peptide- 2 (GLP-2) secretion in healthy adult men (n = 10) in a double-blind placebo-controlled crossover study[8]. Trautvetter and Jahreis[8] suggest that Ca supplementation stimulates the secretion of gastrointestinal hormones (GLP-1 and GLP-2) through the modulation of the intestinal environment. GLP-2 has trophic effects in the intestinal mucosa and influences the tight junction gene expression (occludin and zonula occludens-1 (ZO-1))[90]. On the other hand, Metzler-Zebeli et al.[91] observed a substantial down-regulation of occludin and ZO-1 protein expression in the jejunum of weaned pigs (n = 8 per group) fed high-Ca diets (14.8 g Ca/kg) as compared to adequate Ca diets (8.2 g/kg), whereas gene expression in the colon was unaffected by dietary Ca concentration. The authors suggest that alterations in gene expression were not translated into functional protein, as they did not observe higher intestinal permeability, measured by an enhanced serum acute-phase response or intestinal translocation of LPS[59, 91].

Lipopolysaccharide or serum anti-endotoxin antibodies, gut barrier disintegration and endotoxemia markers, were lower after high Ca diets than after the control diet in mice[51,52] (but not in pigs)[59] (120 mmol Ca/kg [51], 4.8 [52] or 14.8 g/kg diet [52]). Moreover, Ca supplementation reduced bacterial

translocation after Salmonella infection, indicating increased mucosal integrity[27, 38, 50, 52]. However, we emphasize that differences on circulating endotoxin or bacteria may also reflect differences in detoxification or post-absorption clearance. Therefore, it is not related to intestinal translocation only [92, 93].

Metabolic endotoxemia, characterized by moderately elevated serum levels of LPS, is associated with obesity, T2DM, and IR[10, 20]. High fat diets, which are generally associated with obesity, also seem to induce a reduction in intestinal integrity and low-grade endotoxemia[94]. The factors involved in intestinal integrity breakdown in obese patients mainly consist of dysbiosis, adoption of dietary pattern characterized by foods rich in fat and fructose, and deficiencies in the intake of nutrients[95]. In congruence with the mechanisms discussed in this review, we consider that the effects of Ca on intestinal integrity may involve gut microbiota and bacterial fermentation products modulation, in addition to direct action on tight junctions and a decrease in luminal cytotoxicity. For example, butyrate enhances the intestinal barrier because it facilitates the assembly of tight junctions[96]. It is possible that a high Ca (>1100 mg/day in the human studies selected for this review [41, 43]) intake contributes to the maintenance of intestinal integrity, especially in the obese. However, because no human clinical trials have been conducted to date, it is not possible to confirm this association yet.

Conclusions

Dietary Ca appears to positively affect gut microbiota composition and intestinal integrity, which may improve obesity and T2DM treatment. The mechanisms suggested involve BA and FA precipitation and, consequently, a decrease in luminal cytotoxicity, lactobacilli growth, and intestinal mucosal damage reduction. Ca appears to affect colon integrity to a great degree, and the amount of phosphate in the diet or the source of the Ca supplement appears to have minimal effect. PUFA fecal excretion seems to be lower than SFA excretion.

To our knowledge, the contribution of this modulation to the control of obesity and DM is uncertain. Further human clinical trials are needed to explore the potential of dietary Ca or Ca salt supplementation in the modulation of microbiota and intestinal barrier integrity and to ultimately determine the

applicability of relatively simple dietary interventions to the treatment of chronic diseases. Further research is required to define the supplementation period, the dose and the type of Ca supplement (milk or salt) that is more effective in healthy, obese, and diabetic subjects. Since Ca interacts with other components of the diet, these interactions should also be considered in future research. We believe that more complex mechanisms involving extra-intestinal disorders (hormones, cytokines and other biomarkers) also need to be studied.

Acknowledgements

We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). None of the authors had any personal or financial conflict of interest related to this study. The authors' contributions were as follows: J.M.G.G., J.d.A.C. and R.d.C.G.A. designed the concept of the study, and all authors were involved in the literature search and review. J.M.G.G. and J.d.A.C. wrote the manuscript. J.M.G.G., J.d.A.C., and R. d. C. G. A. were involved with editing the manuscript; and all authors read and approved the final manuscript.

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Table 1. Summary of the human and animal studies reviewed

Author, Year	Animals/ Subjects	Intervention	Type/ Source of Ca	Dose of Ca	Period	Main outcomes
Govers & Meer (1993)[40]	6 female Wistar rats/ group	1)Control diet 2, 3 and 4): Low/high Ca diet associated with low/ high phosphate	$CaCO_3$	25, 75, or 225 µmol Ca /g diet; 75, 125, 275 µmol phosphate /g diet.	2 weeks	High-Ca diet (independent of the phosphate content): ↓ cytotoxicity of FW. ↑ fecal excretion of FA. ↓ [BA] and [↓ FA] in the FW. Ca did not change solubility of BA in the ileum, but ↓ 20% solubility of BA in the colon and feces. ↓ solubility of FA in the ileum, colon, and feces.
Lapré et al. (1993)[36]	6 male Wistar rats/ group	1) Control diet (low-Ca diet); 2, 3 and 4) High-Ca diet associated with: butter, saturated margarine, and polyunsaturated margarine	$CaHPO_4$.	25 or 225 µmol Ca/g diet	2 weeks	High-Ca diets: ↓ cytotoxicity of FW, mainly in PUFA-diet. ↑ fecal excretion of BA and FA. ↓[BA] and ↓ [FA] in the LFW. No changes in the fecal pH.
Govers et al. (1994)[37]	7 male Wistar rats/ group	1) Control diet (Low-Ca diet); 2) Dairy Ca; 3) Carbonate Ca; 4) CaP.	Milk Ca; $CaCO_3$; $CaHPO_4$	30 mmol/ kg (control diet) or 150 mmol/kg diet.	2 weeks	All high-Ca diets: ↓ cytotoxicity of FW. ↓ [BA] and ↓ [FA] in the FW. ↓ fecal pH. ↓ cellular damage and epithelium proliferation. ↑ [serum gastrin].
Bovee et al. (1996)[26]	9 pathogen free male Wistar rats/ group	1) Low Ca milk; 2) Milk; 3) Acidified milk; 4)Yoghurt Infection with *Salmonella enteritidis*	Milk, acidified milk and yoghurt	6 or 30 mmol Ca/l	12 days	All high-Ca diets: ↓ cytotoxicity of FW. ↓ [BA] in the FW.
Govers et al. (1996)[41]	13 healthy males (mean age 38 ± 2 y)	1) Control diet (Low-Ca diet). 2) High-Ca diets (Milk and/or yogurt 3% fat, 1 liter/ day, according to personal preference)	Milk and/or yogurt	120 or 1200 Ca mg/day	1 week	All high-Ca diets: ↓ cytotoxicity of FW in 11 of 13 subjects. ↓ [BA] (mainly secondary BA), ↓ [FA] (cytotoxic FA C16-C18 and cholesterol), and [phosphate] in the FW. ↑ fecal pH.

Bovee et al. (1997)[27]	9 males Wistar rats/ group	1) Low-, 2)Medium-, or 3) High-Ca diet. Infection with *Salmonella enteritidis* (after an adaptation period of 11 days)	$CaHPO_4 . 2H_2O$	20, 60, or 180 mmol Ca/kg diet	2 weeks	Medium and High-Ca diet: ↓ cytotoxicity of FW. ↓ [BA] in the FW. ↓ bacterial translocation. No changes in [FA] in the FW.
Bovee et al., (1999)[38]	8 male Wi rats/ group	1) Control diet (Low-Ca diet); 2) High-Ca diet.	$CaHPO_4 . 2H_2O$	20 or 180 mmol Ca/ kg diet	6 days of dietary interven-tion after infection	High-Ca diet: ↓ cytotoxicity of FW. ↓ [FA] and ↓ [BA] in the ileum and in the FW (mainly secondary BA). ↓ diarrhea. ↓ bacterial translocation. ↑ lactobacilli in the ileum and feces before infection) (measured by colony-forming units/g feces).
Ten Brugge ncate et al. (2004)[50]	8 male rats Wistar/ group	1) Control diet (Low-Ca Low-inulin and Low-FOS-diet); 2) Low-Ca High-inulin diet; 3) High-Ca High-inulin diet ; 4) Low-Ca High-FOS diet; 5) High-Ca High-FOS diet. Infection with *Salmonella enteritidis*	$CaHPO_4.2H_2O$	30 (control and low-Ca diets) or 100 mmol Ca/kg diet	6 days	Low-Ca High-inulin and High-FOS diets: ↑ ↓ cytotoxicity of FW and ↑ bacterial translocation after inulin/FOS supplementation – these effects were decreased by high-Ca diets. Low-Ca/ High-inulin and FOS- diets: ↓ fecal pH. ↑ fecal lactobacilli and enterobacteria. High-Ca/High-inulin diet: ↑ fecal lactobacilli High-Ca diets: ↓ fecal enterobacteria (measured by RT-PCR) and ↑ fecal lactate.
Ditschei d et al. (2009)[42]	31 healthy volunteers	1) Placebo (without additional Ca); 2) High Ca-diet. Double-blind, randomized cross-over trial.	$Ca_5(PO_4)_3OH$	1 g Ca/day	4 weeks	High-Ca diet: ↑ fecal excretion of FA. No changes in fecal excretion of BA. ↓ [cholesterol, coprostanol, coprostanone and cholestanol] in the FW.
Rao (2009)[81]	6 male albino rats/ group	1) Low-Ca diet ; 2) High-Ca diet.	Ca carbona te	0.2% or 1.5% Ca	21 days	High-Ca diet: ↑ passive transport of mannitol and Ca and ↓ active transport of Ca by the everted gut sacs.
Schepe ns et al. (2009)[51]	HLA-B27 transgenic rats (n = 9 in control group diet and n=8 in high-Ca group)	1) Low-Ca diet 2) High-Ca diet.	$CaHPO_4$	30 or 120 mmol Ca/kg	7 weeks	High-Ca diet: ↓ cytotoxicity of FW. ↓ [BA] in the FW in week 2 (this effect was not significant in week 6). ↓ diarrhea. ↓ IP (Cr-EDTA). ↓ antibodies against LPS (compared with control diet). ↑ mucosal barrier genes.

Metzler-Zebeli et al. (2010)[57]	8 growing pigs/ group	1) Control diet (soybean) without pectin infusion; 2) Control diet (soybean) with pectin infusion; 3) High-CaP diet without pectin infusion; 4) High-CaP diet with pectin infusion.	Monocalcium phosphate	3 g of phosphate/kg diet (control diets) or 15 g Ca/kg diet	22 days	High Ca-diets: No changes in lactobacilli and total bacteria. ↓ *Enterococcus spp*., *E. faecium* and *C. leptum* in the ileum (measured by RT-PCR). ↑ acetate in the ileum. No changes for Other SCFA (ileum and feces).
Schepens et al. (2010)[67]	13 male Wistar rats/group	1) Control (low-Ca and FOS diet); 2) High-Ca diet; 3) High-FOS diet.	$CaHPO_4.2H_2O$	30 or 120mmol Ca/kg diet	14 days	High Ca-diet: ↑ fecal pH. ↓ enterobacteria fecal (colony-forming units/g faeces). ↓ IP (↓ Cr-EDTA excretion, ↑ lactulose: mannitol ratio, no changes in lactulose excretion.
Van Amptin g et al. (2010)[52]	8 male Wistar rats/ group (Control diet1, n=5)	1) Control diet1: diet without antibiotic and without infection; 2) Control diet 2: diet without antibiotic and with *Salmonella enteritidis* infection; 3) Control diet 3: diet with antibiotic and with infection; 4) High-Ca diet with antibiotic and with infection; 5) High tannic acid diet with antibiotic and with infection.	CaHPO 4.2H2O	1.2 or 4.8g/kg or diet	8 days	High-Ca diet: ↓ cytotoxicity of FW. ↓ diarrhea. No change in bacterial translocation. ↑ fecal lactobacilli (log CFU/g feces). ↓ IP (Cr-EDTA excretion). ↓ anti-LPS antibodies.
Metzler-Zebeli et al. (2011)[58]	8 weaned pigs/ group	1) Control diet (cornstarch casein based diet – low Ca and phosphate); 2) High-CaP diet 3) High-CaP diet + β-glucan ; 4) Low-CaP diet + β-glucan.	Dicalcium phosphate	5.4g or 10g Ca/kg diet	14 days	No changes in gastric, cecal and colonic pHs. High Ca-diets: ↓ gastric streptococci. No changes in lactobacilli (bacterial 16S rRNA genes quantified by qPCR). ↓ gastric lactate and propionate in the large intestine.

Ten Brugge ncate et al. (2011)[56]	8 male Wistar rats/ group	1) Low Ca-diet; 2, 3, 4 and 5) High-Ca diets: CaP; milk Ca; Ca chloride; Ca carbonate Infection with *S. enteritidis* 2 weeks after adaptation. High-fat, Western human-style, purified diet.	$CaHPO_4$, milk Ca (29% Ca), Ca carbonate or Ca chloride	20 or 100 mmol Ca/kg diet	7 days of dietary interventi on after infection	No changes in lactobacilli. High CaP and milk Ca: ↓ enterobacteria All high-Ca diets: ↓ PI induced by *Salmonella.*
Schepe ns et al. (2011)[80]	HLA-B27 transgenic rats (n=9/ group) nontransgenic counterparts (n=7)	1) Control (low Ca and glutathione, Vit C and Vit. E; 2) Low Ca + High glutathione, Vit C and Vit. E ; 3) High Ca + glutathione, Vit C and Vit. E.	$CaHPO_4.2H_2O$	30 (control and low-Ca diets) or 120mmol Ca/kg diet	9 weeks	High Ca-diet: ↓ IP (Cr-EDTA). ↓ diarrhea.
Schepe ns et al. (2012)[47]	10 male Wistar rats	1) FOS + Low-Ca diet + medium phosphate; 2, 3 and 4) FOS + High-Ca diet associated with low-, medium- or high- phosphate.	$CaHPO_4.2H_2O$	30 mmol or 120 mmolCa/kg 35, 70 or 160 mmol phosphate/k g diet.	20 days	High-Ca medium-phosphate diet and High-Ca High- phosphate diet x High-Ca Low-phosphate diet and Low-Ca Low- phosphate diet: ↓ IP (Cr-EDTA excretion). High-Ca diets: prevented the FOS-induced increase in the IP. ↓ cytotoxicity of FW. ↓ cecal lactate. High-Ca High- phosphate diet x Low-Ca Low-phosphate diet : ↑ cecal pH.
Trautve tter et al. (2012)[43]	32 healthy men and women (aged 25±5 y and BMI of 22±3 kg/m^2)	1) Lactobacilli; 2) Lactobacilli + High-CaP; 3) Placebo. Double-blind, placebo-controlled, cross-over trial.	Pentaca lcium hydroxy - triphosp hate ($Ca_5(PO_4)_3OH$)	1g/day	4 weeks (washout: 2 weeks)	High-CaP diet x Control diet and Lactobacilli-diet: ↑ secondary BA fecal excretion. No changes in fecal excretion of FA. Control diet and High-Ca diet: ↑ fecal pH. High-CaP and Lactobacilli-diet (*vs.* placebo): ↑ fecal lactobacilli (qRT-PCR) High-CaP diet: ↓ cholesterol total and LDL-c

Hwang et al. (2013)[88]	5 mice per group: 1) wild-type 2) Calb-9k KO 3) Calb-28k KO 3) Calb-9k/28k KO	1) Normal diet (AIN-76A,) 2) Ca-deficient diet (AIN-76A, 1% phosphate); 3) Ca/ vit D-deficient diet (AIN-76A, 0.35% phosphate)	Ca carbonate anhydrous	1.1% Ca (normal diet) or 0.02% (Ca-deficient diet).	4 weeks	Normal diet: ↑ expression of tight junctions genes in calbindin-9k KO mice *vs.* wild-type ones. Calbindin-9K KO mice fed Ca-Vit. D -deficient diet *vs*. normal diet: ↓ occludin, ZO-*1*, claudin 2 and 15.
Metzler-Zebeli et al. (2013)[59]	8 weaned pigs/ group	1) Wheat-barley diet with adequate CaP content; 2) Wheat-barley diet with high CaP content; 3) Corn diet with adequate CaP content ; 4) Corn diet with high CaP content	Monocalcium phosphate	8.2 or 14.8 g Ca/kg diet.	14 days	No changes in gastric, ileal, colonic, and fecal pHs. No changes in serum LPS and fecal LPS. High-CaP diets: ↑ gastric *Enterobacteriaceae* and ileal *Enterococcus*, *Bacteroides-Prevotella-Porphyromonas*, and *Campylobacter*. ↓ caproate in the colon (qPCR). High CaP level increased total SCFA, acetate, and isovalerate in gastric digesta, when combined with the wheat-barley diet but not with the corn diet.
Overduin et al. (2013)[70]	7 or 8 pathogen-free Wistar rats/ group	1) Low-Ca low-GOS diet; 2) High-Ca low-GOS diet; 3) Low-Ca high -GOS diet; 4) High-Ca high-GOS diet.	Ca monophosphate	30 or 100 Ca mmol/kg diet; 0 or 6% GOS of dry weight.	3 weeks	High-GOS diets: ↑ lactic acid levels and ↓ butyric acid in proximal colonic contents; High-Ca diets: abolished the GOS-related elevation of lactic acid, while increasing propionic acid levels.
Trautvetter et al. (2014)[8]	10 healthy men (aged 27 $\pm$ 4y and mean BMI of 23.1 $\pm$ 2.3 kg/m^2)	1) Placebo; 2) High-CaP diet. Double-blind, placebo-controlled, cross-over trial.	$Ca_5(PO_4)_3OH$	1 g/day	3 weeks (washout: 2 weeks)	↑ AUC of GLP-1 (total and active) and GLP-2 after the repeated CaP administrations x placebo. No changes in insulin and glucose.

Metzeler-Zebeli et al. (2015)[91]	8 weaned pigs/ group	1) Wheat-barley diet with adequate CaP content; 2) Wheat-barley diet with high CaP content; 3) Maize diet with adequate CaP content ; 4) Maize diet with high CaP content.	Monocalcium phosphate	8.2 or 14.8 g Ca/kg diet.	15 days	High-Ca diets *vs.* adequate Ca diets: ↓ jejunal ZO-1and occludin expression.
Zheng et al. (2015)[44]	15 healthy young men (ages, 18−50 years; BMI 20−28 kg/m2)	1) Control diet; 2) High in semiskimmed milk; 3) High in semihard cow's cheese with equal amounts of dairy calcium.	Dairy Ca	0 (control diet) or 1.7 g/day.	14 days	Cheese and milk consumption: ↑ fecal excretion of acetate, propionate, and lipid; Cheese *vs.* milk consumption: ↑ butyrate, hippurate, and malonate.

AUC, area under the curve; BA, bile acid; BMI, body mass index; Ca, calcium; Calb, calbindin; CaP, calcium phosphate; FA, fatty acid; FOS, fructooligosaccharides; FW, fecal water; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; GOS, galacto-oligosaccharides; IP, intestinal permeability; KO, Knouckout; LDL-c, Low-density lipoprotein- cholesterol; LPS, lipopolysaccharide; PUFA, polyunsaturated fatty acids; qPCR, quantitative PCR;
qPCR, quantitative polymerase chain reaction; RT-PCR, Real-time polymerase chain reaction; SCFA, short-chain fatty acids; Vit., vitamin; ZO-1, zonula occludens 1; ↓, decrease; ↑, increase; [ ], concentration

2.2. REVIEW ARTICLE 2 - Published in Metabolism (2017), 68, 133–144. DOI: 10.1016/j.metabol.2016.12.009)

Title:

Metabolic Endotoxemia and Diabetes *Mellitus*: a Systematic Review

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Short-title: Endotoxemia and diabetes

Abstract

In this systematic review we analyzed studies that assessed serum concentrations of lipopolysaccharide (LPS) and/or lipopolysacharide-binding protein (LBP) in diabetic patients compared with healthy people. Articles were selected using PubMed and Scopus. Search terms used were endotoxemia, endotoxins, LPS, LBP, diabetes mellitus (DM), type 1 (T1DM), type 2 (T2DM), insulin resistance, humans, epidemiologic studies, population-based, survey, representative, cross-sectional, case–control studies, observational, and clinical trials. Two authors independently extracted articles using predefined data fields, including study quality indicators. There was a great variability in the estimates of metabolic endotoxemia among the studies. Most of the studies observed higher LPS or LBP concentrations in diabetic subjects than in healthy controls. T1DM and T2DM subjects presented higher mean fasting LPS of 235.7% and 66.4% compared with non-diabetic subjects, respectively. Advanced complications (e.g. macroalbuminuria) and disease onset exacerbate endotoxemia. Antidiabetic medications decrease fasting LPS concentrations. Among these medications, rosiglitazone and insulin present higher and lower effects, respectively, compared with other treatments. T1DM and T2DM seem to increase metabolic endotoxemia. However, some confounders such as diet, age, medication, smoking and obesity influence both diabetes and endotoxemia manifestation. A better understanding of the interaction of these factors is still needed.

Keywords: Endotoxin, Lipopolysaccharide, Lipopolysaccharide-binding protein, Diabetes Mellitus, Insulin Resistance

1. Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [1]. Type 1 diabetes (T1DM) results from beta-cell destruction, usually leading to absolute insulin deficiency [1]. Type 2 diabetes (T2DM) occurs due to the progressive loss of insulin secretion and/or insulin action, usually with a contribution from insulin resistance (IR) [1]. The prevalence and incidence of DM have increased during recent decades, especially in Western countries [1]. Short and long-term complications due to uncontrolled glycemia lead to high human, social, and economic burdens [1]. Therefore, understanding the features involved in the pathophysiology of DM is of considerable value to treat DM and prevent its progression.

Increased intestinal permeability may contribute to low-grade inflammation, leading to insulin resistance, and DM [2]. The intestinal epithelial monolayer is an important barrier between the organism and the external environment [3]. A healthy intestinal barrier allows the passage of water, nutrients and bioactive compounds, and avoids the passage of harmful substances such as microbial and dietary antigens [3]. Evidence, largely from animal studies, indicates that DM favors endotoxin (especially lipopolysaccharide (LPS)) translocation across the intestinal barrier, leading to its mild increase in concentration in the bloodstream [4]. LPS is the major component of the outer membrane of the Gram-negative bacteria. This endotoxin is composed of three modules: a highly variable O-antigen constituted of repeating oligosaccharide units, a core oligosaccharide and lipid A [5]. Lipid A component is responsible for much of LPS toxicity. Toll-like receptors (TLR) of the innate immune system recognize lipid A and then trigger immune and inflammatory responses [5].

Integrity breakdown and increased intestinal permeability favor LPS translocation from the intestinal lumen to the bloodstream, causing metabolic endotoxemia [2,4]. LPS has a short half-life, so LPS-binding protein (LBP) has been used as a metabolic endotoxemia marker [6,7]. LBP is an acute-phase protein synthesized in the liver [6,7]. The binding of LBP–LPS complex to cluster of differentiation 14 (CD14), which is mainly expressed by macrophages

and neutrophils, mediates signal transduction, including nuclear factor kappa B (NF-κ B) activation via TLR4, leading to the activation of innate and adaptive inflammatory responses [6,7]. Considering that LBP represents the innate immune response triggered by LPS, assessing LBP concentrations is an indirect way to evaluate active LPS. Consequently, LBP is a good marker of metabolic endotoxemia [6,7].

Animal and human studies indicate LPS as an antigen that activates the immune system, playing an important role in the pathogenesis of metabolic chronic diseases related to subclinical inflammation, such as obesity, IR, T2DM, and dyslipidemia [2,8,9]. However, the influence of LPS concentrations on glucose homeostasis in humans is not well understood. In this context, new links between endotoxemia and DM should be highlighted to better treat and prevent DM complications. Therefore, in this systematic review we examined the studies that assessed serum concentrations of LPS and/or LBP in diabetic patients compared with healthy controls. We also discuss existing evidence for the proposal of possible mechanisms linking metabolic endotoxemia and DM.

2. Methods

2.1. Protocol and Registration

This systematic review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [10] (**S1 Appendix** - Checklist) and was registered in PROSPERO (registration number: CRD42015020532).

2.2. Literature search

Two authors (JMGG and JAC) independently searched for original articles on endotoxemia status in diabetes mellitus type 1 (DM1), DM2 or impaired glucose tolerant (IGT) patients in the following electronic databases: PubMed (www.pubmed.com) and Scopus (www.scopus.com). Keywords were chosen from the Medical Subject Headings terms using the following search strategy: (Endotoxemia OR Endotoxins OR Lipopolysaccharides or Lipopolysaccharide-binding protein) AND (Diabetes Mellitus, Type 2 OR Diabetes Mellitus, Type 1 OR Insulin Resistance) AND humans AND

(epidemiologic studies OR population-based OR survey OR representative OR cross-sectional OR case–control studies OR observational OR clinical trials) NOT (reviews).

The search strategies had no date restrictions and included articles published in English, Portuguese and Spanish. The date last searched was October 30, 2016. References from the extracted articles were also consulted to complete the data bank.

2.3. Studies selection

We included all published randomized controlled trials (RCTs), cross-sectional and cohort studies comparing fasting plasma LPS or LBP concentrations in diabetic human patients versus healthy non-diabetic controls (at baseline). Studies were included in the present review if they met the following criteria defined a priori: (1) Population: T1DM, T2DM or IR subjects; (2) Control group: non-diabetic healthy subjects; (3) Exposure: presence of T1DM, T2DM or IR; (4) Main outcomes: report of mean or median plasma LPS or LBP concentrations; (5) Study design: cross-sectional comparison of endotoxemia; (6) Measurement of circulating LPS concentrations by chromogenic kinetic limulus amebocyte assay (LAL assay) or LBP concentrations by enzyme-linked immunosorbent assays (ELISA).

We excluded reviews, case reports, letters, commentaries, abstracts, and unpublished articles. We excluded studies that did not have a control group (healthy non-diabetic subjects), did not include diabetic patients or RI or those that did not discriminate diabetic subjects compared with controls, animal studies, studies that did not indicate LPS values or in which LPS values were not adequately described (e.g. only in graphs, only correlation data), studies with LPS infusion, in vitro assays and other systemic diseases other than diabetes and obesity (e.g. metabolic syndrome, hypertension, periodontitis and AIDS).

2.4. Data extraction

All studies were independently screened and evaluated for selection by two authors (JMGG and JAC). After all abstracts were reviewed, data

comparisons between investigators were conducted to ensure completeness and reliability. We did not contact authors of the original articles in the case of missing data. The inclusion criteria were independently applied to all identified studies. Differing decisions were resolved by consensus. For each included article, we extracted information of the title, authors, publication year, name of the study, type of study, study aim, sample size, number of diabetics and control subjects, study design, proportion of men, mean age, mean body mass index (BMI, calculated as body weight in kilograms divided by height in meters squared), mean HbA1c or fasting glucose, mean/median LPS or LBP concentrations, and assay used for measuring LPS or LBP concentrations.

2.5. Assessment of Reporting Quality and Risk of Bias

We assessed study quality using data reported in each study on representativeness, validity and reliability. A study was considered representative if (1) this feature of the study was explicitly addressed in the corresponding full-text article or (2) any statement made by the authors suggested that the actual sample reflected the target population (e.g., sample size calculation, description of inclusion and exclusion criteria, etc.). A study was classified as non-representative if the article's corresponding full-text contained information about an existing selection bias. Assessment validity was evaluated using information about LPS or LBP measure (e.g. detailed information about the method of assessment under nonpyrogenic conditions or citation of a study in which that was previously detailed). Finally, a study was classified as reliable if the intra and inter-assay coefficients of variation (CV) were below 10 and 15 %, respectively. In instances where details about representativeness, validity or reliability were not provided, we created a separate category ('unknown') for each quality criterion.

Two authors (JMGG and JAC) assessed risk of bias for each study using predefined criteria described by the Agency for Healthcare Research and Quality "Methods Guide for Effectiveness and Comparative Effectiveness Reviews" [11], using questions specified in the RTI Item Bank [12] and the Cochrane Risk of Bias tool [13]. We selected items based on relevance to the topic and anticipated sources of bias. We assessed the potential for selection

bias, performance bias, attrition bias, detection bias, and reporting bias. The tool presents design-specific criteria to assess risk of bias, with 12 questions for RCT, 13 for cohort, and 9 for cross-sectional studies [11]. Studies were classified as having a low risk of bias when > 80% questions were answered as ‘‘yes (low risk)’’, a moderate risk of bias when 50 to 79% of the questions were answered as ‘‘yes (low risk)’’ and a high risk of bias when < 50% questions were answered as ‘‘yes (low risk)’’. Discrepant opinions between authors were resolved by consensus.

2.6. Data analyses

A statistical meta-analysis was not justified because of the marked heterogeneity of the included studies. We followed guidance from the Cochrane handbook, which supports the use of a systematic, narrative approach when a meta-analysis is inappropriate [13].

We summarized all studies reviewed in this paper in tables according to main characteristics and results from single studies, geographic distribution, quality assessment, risk of bias, and correlation between LPS or LBP concentrations and other variables. The studies were arranged chronologically by year of publication, beginning with the last published study. The primary outcome measure the difference in LPS or LBP fasting plasma mean (or medians) comparing diabetic subjects to non-diabetics controls. We also calculated the percentage change in LPS or LBP concentrations comparing cases and controls. We reported the variables that significantly correlated with LPS or LBP according to the information provided by the authors, highlighting the positive and negative correlations, and we described the type of statistical analyses used by the authors.

3. Results

3.1. Study selection

We identified a total of 867 studies after searching the two databases. A total of 331 duplicates were removed resulting in 536 unique records after which 506 studies were excluded based on their titles and abstracts because they were considered irrelevant to the topic of interest. After reading the full text of

the remaining 30 studies, 14 met all the criteria for the systematic review. The most common reasons for exclusion were animal, duplicate , *in vitro* or LPS infusion studies, language (Chinese, Russian, and Ukrainian), lack of a comparison group (healthy control), lack of diabetics or insulin resistant patients, presence of participants with multiple diseases, and absence of LPS or LBP as outcome (**Figure 1**).

3.2. Description of Included Studies

The fourteen studies included in the present review (**Table 1**) contained data on a total of 9.773 subjects (7.253 non-diabetic controls, 72 IGT patients, 2.424 diabetics patients (1.183 T1DM and 1.241 T2DM subjects), and 198 overweight or obese non-diabetic subjects) [6, 14-26]. These studies had sample sizes ranging from 30 [24] to 6.632 [19] participants with a median of 94 (interquartile range 75.7 – 340). Ten studies (71.4%) included T2DM subjects [6, 14, 15, 19-21, 23-26], and four studies (28.6%) included T1DM subjects [16-18, 22]. Two studies (14.3%) also evaluated impaired glucose tolerant subjects [6, 20], and four (28.6%) discriminated non-diabetic overweight subjects [17, 20, 21, 25]. While the majority of studies contained data on males and females [14-20, 22- 24], three studies (21.4%) contained data on only females [21, 25, 26], and another one (7.1%) was restricted to males [6]. The overall proportion of males was 42%. The mean age was 37.3 $\pm$ 4.9 years for T1DM subjects (*vs.* 37.7 $\pm$ 5.9 years for healthy controls), and 51 $\pm$ 4.8 years for T2DM subjects (*vs.* 42.1 $\pm$ 8.4 years for healthy controls). In terms of geographic distribution, most studies were conducted in Europe and Asia (England, n =1; France, n=1; Finland, n =3; India, n=1; Russia, n=1; Saudi Arabia, n =3; Spain, n=1; United Kingdom, n=1) [6, 14, 15, 17-20, 22-26] . One study was conducted in Africa [21] and another in the United States [16] (**Table 2**). The studies included nine cross-sectional studies [6, 15-18, 21-24], three clinical [20, 25, 26] trials and two cohort studies [14, 19] that compared fasting serum LPS or LBP concentrations of diabetic subjects *vs.* healthy controls. Only baseline data from the clinical trials and cohorts were used (**Table 2**).

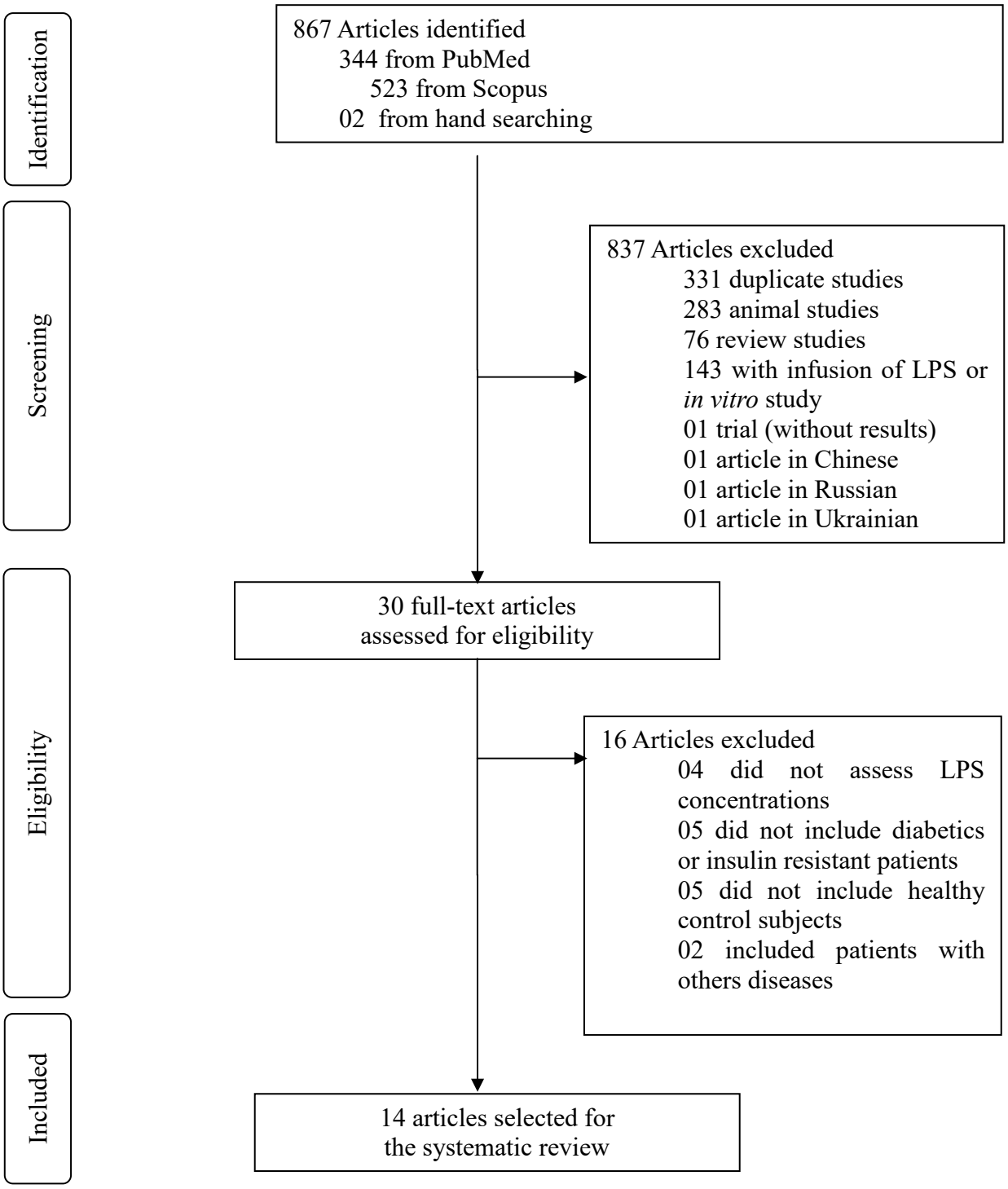

Figure 1. Flowchart of study-selection process.
PubMed, www.ncbi.nlm.nih.gov/pubmed/; Scopus, www.scopus.com
LPS: lipopolysaccharide. Hand searching was conducted on the references lists of papers included in the systematic review.

Table 1. Characteristics and main results from single studies on LPS or LBP levels in diabetic subjects and healthy controls

Author (year)	Study aim	Sample size	Males (%)	Age (years)	BMI (kg/m^2)	HbA1c (%) or glycemia (mmol/L)[2]	Fasting endotoxin (EU/mL) or LBP (µg/ml)1 levels at baseline	% change in LPS or LBP levels *vs.* ND
Gubern et al. [6][1]	To associate bactericidal/ permeability-increasing protein to insulin sensitivity.	114 ND	100	46.2 ± 11.9[a]	27.01±3.6[a]	4.78 ± 0.34[a]	27.82 (9.8, 50.1)[a]	
		60 IGT		53.2 ±11.2[bc]	29.5 ± 3.9[b]	5.0 ± 0.58[b]	17.26 (9.59, 44.93)[ab]	↓ 37.9%
		170 T2DM		57.2 ± 11.8[c]	32.3 ± 7.0[ab]	7.3 ± 1.7[b]	65.03 (57.9, 72.08)[b]	↑ 133.7%
Creely et al. [14][2,3]	To examine circulating LPS in T2DM subjects.	25 ND	80	48.1 ±19.2[a]	29.5 ± 4.3[a]	5.6 ± 0.9[a]	3.1 (1.7)[a]	
		25 T2DM		52.2 ±11.7[a]	31.8 ± 4.5 [a]	8.6. ± 2.5[b]	5.5 (1.6)[b]	↑ 77.4%
Attas et al. [15][2,4]	To investigate the relationship between endotoxin and various metabolic parameters of diabetic patients.	67 ND	49	44.1 ± 9.9[a]	30.0 ± 5.2[a]	5.5 ± 1.5[a]	4.2(3.1–5.1)[a]	
		346 T2DM treated with:						
		36 diet-controlled		48.3 ± 9.[b]	29.6 ± 5.8[a]	7.1 ± 2.8[d]	7.9(5.7–10.0)[b]	↑ 88%
		141 met		53.0 ± 10.5[cd]	32.0 ± 5.8[a]	9.6 ± 3.4[cd]	7.5(4.6–8.7)[c]	↑ 78.6%
		22 RSG		52.3 ± 9.5[bd]	29.6 ± 5.8[a]	8.4 ± 1.9[bd]	5.6 (4.2–6.1)[c]	↑ 33.3%
		100 met/RSG		52.5 ± 9.0[cd]	31.0 ± 5.3[a]	9.4 ± 3.9[cd]	7.4 (4.8–9.6)[b]	↑ 76.2%
		47 insulin		55.6 ± 11.4[d]	29.0 ± 6.2[a]	9.5 ± 3.8[d]	9.2 (6.6–10.7)[b]	↑ 119%
Devaraj et al. [16][5]	To examine circulating levels of TLR2 and TLR4 ligands in T1DM compared with matched healthy controls.	37 ND	51.4	34 ± 11 [a]	24 ± 4[a]	5.4 ± 0.4[a]	2.53 ± 0.67 [a]	
		34 T1DM	44.1	32 ± 11[a]	25 ± 4[a]	7.9 ± 1.4[b]	3.32 ± 0.82[b]	↑ 31.2%

(Continued)

Table 1. Characteristics and main results from single studies on LPS or LBP levels in diabetic subjects and healthy controls

Author (year)	Study aim	Sample size	Males (%)	Age (years)	BMI (kg/m^2)	HbA1c (%) or glycemia (mmol/L)[2]	Fasting endotoxin (EU/mL) or LBP (µg/ml)1 levels at baseline	% change in LPS or LBP levels *vs.* ND
Lasseniu s et al. [17]	To investigate whether serum LPS is associated with the components of the MetS in T1DM patients.	345 ND:	51.3	33 ± 10	24.3 ± 3.6	NA	61 (44, 79)	Only T1DM patients with macroalbuminuri a showed higher LPS levels than ND subjects (↑ 9.8%).
		219 lean		33 ± 10[a]	22.2 ± 1.7[a]		60 (44, 80)[a]	
		126 overweight		33 ± 9[a]	28.2 ± 2.8 [b]		62 (49, 82)[b]	
		904 T1DM:	47.8					
		587 normal AER		44 (36, 53)[a]	25.6 ± 4.2[a]	7.7 ± 1.3[a]	57 (50, 69)[a]	
		144 with microalbuminuria		46 (37, 55)[ab]	26.4 ± 4.2[b]	7.8 ± 1.8[a]	56 (47, 72)$_a$	
		173 with macroalbuminuria		48 (40, 56)[b]	27.0 ± 4.9[b]	7.8 ± 2.0[a]	67 (52, 96)[b]	
Okoroko v et al. [18]	To determine the possible role of the excess of LPS on T1DM onset.	50 ND	58	11.14 ± 0.57	NA	4.56 ± 0.04	0.4 ± 0.03[a]	
		45 T1DM:	53.3					
		15 T1DM (onset)		6.6 ± 1.12		8.1 ± 0.59	3.93 ± 0.79[b]	↑ 882%
		30 T1DM (>2 y of DM)		12.3 ± 0.71		8.73 ± 0.33	2.37 ± 0.27[c]	↑ 492%
Pussinen et al. [19][4]	To investigate whether endotoxemia and incident diabetes are associated.	6170 ND	50.5	53.2 ± 11.0[a]	26.7 ± 4.1[a]	NA	61.06 ± 36.11[a]	↑ 26.2%
		462 T2DM	60.1	57.3 ± 9.4[b]	31.6 ± 5.2[b]		77.03 ± 42.03[b]	
Harte et al. [20][4]	To evaluate the changes in circulating endotoxin after a high-saturated fat meal.	9 ND lean	62.9	39.9 ± 11.8[a]	24.9 ± 3.2[a]	5.9 ± 0.31%[a]	3.3 ± 0.15[a]	
		15 ND obese		43.8 ± 9.5[a]	33.3 ± 2.5[b]	5.9 ± 0.49[a]	5.1 ± 0.94[a]	↑ 54.5%
		12 IGT		41.7 ± 11.3[a]	32.0 ± 4.5[b]	6.3 ± 0.47[b]	5.7 ± 0.1[b]	↑ 72.7%
		18 T2DM		45.4 ± 10.1[a]	30.3 ± 4.5[c]	7.5 ± 1.12%[b]	5.3 ± 0.54[b]	↑ 60.6 %

(Continued)

Table 1. Characteristics and main results from single studies on LPS or LBP levels in diabetic subjects and healthy controls

Author (year)	Study aim	Sample size	Males (%)	Age (years)	BMI (kg/m^2)	HbA1c (%) or glycemia (mmol/L)[2]	Fasting endotoxin (EU/mL) or LBP (µg/ml)1 levels at baseline	% change in LPS or LBP levels *vs.* ND
Hawkesworth et al. [21][2]	To investigate metabolic endotoxemia in Gambian women.	31 ND lean	0	41.5 ± 6.2	20.8 ± 1.8	5.0 (4.8, 5.2)	3.89 (3.20, 4.73)[a]	
		33 ND obese		43.4 ± 5.4	34.3 ± 4.5	5.5 (5.2, 5.8)	3.86 (3.30, 4.52)[a]	
		29 obese T2DM		45.1 ± 5.2	33.3 ± 5.7	9.2 (7.7, 10.9)	5.19 (3.43, 7.87)[b]	↑ 33.4%
Peraneva et al. [22]	To detect serum bacterial DNA in subjects with high LPS activity.	200 ND	48.5	46 ± 12[a]	25.9 ± 3.8[a]	7.8 ± 1.0[a]	66 (54, 93)[a]	
		200 T1DM	47	36 ± 11[b]	23.8 ± 2.8[b]	5.1 ± 0.3[b]	55 (42, 71)[b]	↑ 20%
Jayashree et al. [23][6]	To compare serum LPS levels in T2DM patients *vs.* healthy controls.	45 ND	55.5	46 ±9[a]	26.9 ± 3.9 [a]	5.6 ± 0.4[a]	0.47 ± 0.02[a]	
		45 T2DM	51.5	51 ± 6[b]	27.2 ± 6.0[a]	8.0 ± 2.2[b]	0.57 ± 0.028[b]	↑ 21.3%
Verges et al. [24]	To evaluate lipoprotein kinetics and plasma LPS distribution.	14 ND	64	29.6 ± 11.5[a]	22.4 ± 1.8[a]	NA	0.94 ± 0.66[a]	No significant difference
		16 T2DM	31	55.8 ± 9.2[ab]	31.8 ± 4.0[b]	7.4 ± 1.5	0.92 ± 0.66[a]	
Al-Disi et al. [25]	To determine the influence of a high-fat meal on changes in endotoxin levels.	18 ND	0	24.4 ± 7.9 [a]	22.2 ± 2.2[a]	4.8 ± 0.9[a]	1.5 ± 0.1[a]	
		24 overweight/ obese	0	32.0 ± 7.8[b]	28.5 ± 1.5 [b]	4.7 ± 0.4[a]	3.0 ± 0.5[b]	↑ 100%
		50 T2DM	0	41.5 ± 6.2[c]	35.2 ± 7.7[c]	7.9 ± 2.7[b]	3.4 ± 0.8[b]	↑ 126.7%
Zaman and Zaman [26]	To assess postprandial endotoxemia in nonobese postmenopausal women and diabetic patients.	80 ND	0	48 ± 5	23 ± 1.4	5.1 ± 0.6	0.37 ± 0.02	No significant difference
		80 T2DM	0	48 ± 6	24 ± 2.0	9.1 ± 2.1	0.39 ± 0.03	

Data are means ± SD or median (interquartile range). Different letters indicate significant differences between groups in the same study. [1] Study that evaluated only LBP levels. [2] Studies that evaluated glycemia (not HbA1c levels). 3 Endotoxin, inv log EU/ml (geometric mean). [4] Endotoxin Log transformed before comparisions. [5] Endotoxin (nmol/l). [6] Endotoxin (µg/ml).
Abbreviation: AER, Albumin Excretion Rate; HOMA-IR, Homeostasis Model Assessment-Estimated Insulin Resistance; IR, insulin resistance; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharide; Met, Metformin; MetS, Metabolic Syndrome; MVC, Microvascular Complications; NA, Not Available; ND, Non-Diabetic subjects; RSG, Rosiglitazone; T1DM, Type 1 Diabetic subjects; T2DM, Type 2 Diabetic subjects.

Table 2. Geographic distribution, type of study, quality assessment and risk of bias of the selected studies

Author, year	Country	Type of study	Representativeness	Measurement validity	Reliability	Overall risk of bias
Gubern et al.(2006) [6]	Spain	Cross-sectional	Yes	Yes	Yes	Low risk (8/9)
Creely et al. (2007) [14]	England	Cohort	Unknown	Unknown	Yes	Moderate risk (9/13)
Attas et al. (2009)[15]	Saudi Arabia	Cross-sectional	Yes	Yes	Unknown	Moderate risk (7/9)
Devaraj et al. (2009) [16]	United States of America	Cross-sectional	Yes	Unknown	Unknown	Moderate risk (7/9)
Lassenius et al. (2011) [17]	Finland	Cross-sectional	Yes	Unknown	Yes	Low risk (8/9)
Okorokov et al. (2001) [18]	Russia	Cross-sectional	Unknown	Unknown	Unknown	High risk (4/9)
Pussinen et al. (2011) [19]	Finland	Prospective cohort	Yes	Yes	Unknown*	Moderate risk (9/13)
Harte et al. (2012) [20]	United Kingdom	Clinical trial	Yes	Yes	Yes	Low risk (10/12)
Hawkesworth et al. (2013) [21]	Africa	Cross-sectional	No	Yes	Unknown	High risk (4/9)
Peraneva et al. (2013) [22]	Finland	Cross-sectional	Yes	Yes	Unknown	Moderate risk (6/9)
Jayashree et al. (2014) [23]	India	Cross-sectional	Unknown	Unknown	Yes	Moderate risk (6/9)
Verges et al. (2014) [24]	France	Cross-sectional	Yes	Yes	Unknown	Moderate risk (6/9)
Al-Disi et al. (2015) [25]	Saudi Arabia	Clinical trial	Yes	Yes	Yes	Moderate risk (9/12)
Zaman and Zaman (2015) [26]	Saudi Arabia	Clinical trial	Yes	Yes	Yes	High risk (6/12)

* Only interassay coefficient of variation is showed.

3.3. Quality assessment and risk of bias

In terms of study quality, ten studies (71.4 %) were classified as representative of the target population [6, 15-17, 19, 20, 22, 24-26]. One study (7.1%) was qualified as non-representative according to the criteria defined previously [21]. Evidence of representativeness could not be established in three (21.4%) studies due to missing information [14, 18, 23]. Assay validity was reported in nine studies (63.4%) [6, 15, 19-22, 24-26]. Information on assay reliability was provided in seven studies (50%) [6, 14, 17, 20, 23, 25, 26], and all of them were classified as providing reliable LPS or LBP measurements (**Table 2**). Three (21.4%) studies were considered as having a high risk of bias [18, 21, 26], eight (57.1%) were classified as having a moderate risk of bias [14-17, 19, 22-25] and three (21.4%) were considered as having a low risk of bias [6, 17, 20]. The major limitations were in the selection bias (inclusion and exclusion criteria not clearly defined or uniformly reported to all comparison groups (nine studies, 64.3%)), and lack of control or adjustment for confounding variables (seven studies, 50%)).

3.4. Results of individual studies

There was a great variability in the estimates of metabolic endotoxemia among the studies. The mean and median values of LPS in non-diabetic subjects ranged from 0.37 [26] to 61.06 EU/ml [10], and from 3.89 [21] to 66 EU/ml [22], respectively (considering only studies that used the unit EU/ml). Among diabetic subjects, mean and median LPS concentrations ranged from 0.39 (T2DM subjects) [26] to 77.03 EU/ml (T2DM subjects) [19] and from 5.19 (obese T2DM subjects) [21] to 67 EU/ml (T1DM subjects with macroalbuminuria) [17], respectively (considering only studies that used the unit EU/ml).

Higher LPS or LBP concentrations in diabetic subjects compared with healthy controls (**Table 1**) were observed in most studies. T1DM and T2DM subjects presented higher mean fasting LPS of 235.7% and 66.4% compared with non-diabetic subjects, respectively. Significant differences in LPS concentrations between T2DM subjects compared with healthy controls were not detected in only two studies [24, 26]. LPS concentrations in T1DM subjects

with microalbuminuria was not different when compared to control subjects instead of T1DM subjects with macroalbuminuria [17]. Considering the studies in which LPS concentrations were statistically different between diabetic and control subjects, the lowest difference was observed in T1DM patients with advanced kidney disease (LPS concentrations 9.8% higher) [17] and a greater difference in T1DM subjects at the disease onset (LPS concentrations 882% higher) [18]. Among the T2DM subjects, the lowest difference compared with control subjects was observed in the study of Jayashree et al. [23] (LPS concentrations 21.3% higher) and the greatest difference among diabetic women in the study of Al-Disi et al. [25] (LPS concentrations 126.7% higher).

Although in the study of Verges et al. [24] fasting LPS concentrations in T2DM patients were not different from the control subjects, the postprandial LPS distribution in the two groups was different. T2DM subjects had higher LPS-very low-density lipoprotein (VLDL), LPS-high-density lipoprotein (HDL), free (nonlipoprotein bound) LPS and lower LPS-low-density lipoprotein (LDL) [24]. In another study in which fasting plasma LPS did not differ in T2DM patients and controls, diabetics had higher increase in LPS concentrations four hours after a meal [26]. However, the authors did not describe the type of meal provided to the participants (e.g. high-fat meal) [26]. Similarly, the other two clinical trials included in this review observed higher increase in postprandial LPS concentrations in T2DM patients compared with healthy controls after the consumption of a meal containing 75 grams of fat [20, 25].

Gubern et al. [6] verified higher LBP concentration and lower bactericidal/ permeability-increasing protein (BPI) in T2DM patients and subjects with impaired glucose tolerance compared with non-diabetic subjects. BPI competes with LBP for the binding of endotoxin, but BPI-LPS complexes (in contrast to LBP and LPS) do not activate immune response. Interestingly, the treatment with metformin increased BPI concentrations in T2DM patients, although their values remained lower than the control ones [6].

Other studies also reported the influence of antidiabetic medication on endotoxemia. Rosiglitazone (RSG) decreased fasting serum insulin and plasma LPS in T2DM subjects [14, 15]. Creely et al. [14] detected lower LPS concentrations in T2DM subjects who were treated with oral hypoglicemics

and/or insulin (n=14) compared with those treated with diet alone (n=11). Attas et al. [15] examined LPS concentrations in non-diabetic subjects (n=67) and T2DM subjects treated with: diet-controlled (n=36), metformin (n=141), RSG (n=22), combined fixed dose of metformin/RSG (n=100), and insulin (n=47). LPS concentrations were higher in T2DM subjects compared to non-diabetic ones. Among T2DM subjects, those who were treated with RSG and insulin had lower and higher LPS concentrations, respectively, compared with other treatments.

In general, the most cited variables that correlated with LPS or LBP concentrations were triglycerides [6, 15, 17, 19, 20, 23, 25], fasting glycemia [6, 15, 21, 23], insulinemia [6, 14, 15, 17], CRP [6, 17, 19, 22], HbA1c [6, 16, 23], and total cholesterol [15, 19, 25] (**Table 3**). In five studies separate clinical variables for diabetic and non-diabetic participants were correlated. Considering only diabetic subjects, insulinemia [6, 14, 15, 17], trygliceridemia [6, 15, 17, 25], glycemia [6, 15], and HDL [6, 15] were the most cited variables that correlated with LPS or LBP concentrations. Among control subjects, insulinemia [14, 17], and trygliceridemia [15, 25] were the most cited. The studies of Okorokov et al. [18] and Zaman & Zaman [26] did not correlate LPS with other variables.

Because HDL is the major factor involved in endotoxin neutralization, some authors used the LPS/HDL ratio as a functional measure of LPS activity [17, 19]. LPS/HDL ratio was associated with increased risk of incident diabetes and was also correlated with metabolic syndrome components [17, 19]. The risk was independent of other risk factors for DM (blood glucose, lipids, CRP, BMI, etc.) and was also independent of other factors that affect endotoxemia (cholesterol, HDL, smoking, etc.) [17, 19].

Table 3. Variables that significantly correlated with LPS or LBP concentrations/activity

Author (year)	Type of analyses	Main clinical variables that correlated with LPS or LBP concentrations	
		Non-diabetic subjects	**Diabetic subjects**
Gubern et al. [6]*	Pearson's correlation analysis	Positive: BMI (r = 0.13) Negative: BPI (r = -0.31)	Positive: BMI (r = 0.4), glucose (r = 0.37), HbA1c (r = 0.35), insulin (r = 0.30), TG (r = 0.31), CRP (r = 0.33) Negative: HDL (r = -0.21)
Creely et al. [14]	Pearson's correlation analysis	Positive: Insulin (r = 0.68); HOMA-IR (r = 0.69)	Change in insulin levels after RSG treatment (r = 0.673)
Attas et al. [15]	Multiple regression analysis	Positive: TG (R^2 = 0.192); total cholesterol (R^2 = 0.163)	Positive: TG (R^2 = 0.42); total cholesterol (R^2 = 0.10), glucose (R^2 = 0.076) and insulin (R^2 = 0.032) Negative: HDL-cholesterol (R^2 = 0.055)
Devaraj et al. [16]	Spearman's rank Correlation	**All subjects:** Positive with TLR4 (r = 0.56); HbA1c (= 0.64)	
Lassenius et al. [17]	Multivariate linear regression analyses Pearson's correlation analysis	Positive: TG (β=0.69), diastolic blood pressure (β = 0.10); TG (r = 0.396); Insulin (r = 0.312), CRP (r = 0.272) Negative: age at onset of diabetes (β = -0.14) **LPS activity**:** Positive correlation with HOMA (r = 0.121); TG (r = 0.505); insulin (r = 0.370); CRP (r = 0.331); BMI (r = 0.199)	Positive: ThG (r = 0.73) HOMA (r = 0.213), Insulin (r = 0.25) ; TG (r = 0.325) (patients with IgAGN) **LPS activity**:** Positive correlation with HOMA (r = 0.230); TG (r = 0.496); insulin (r = 0.251); BMI (r = 0.343) (patients with IgAGN)
Pussinen et al. [19]	Two-tailed Pearson correlation	**All subjects:** Positive with CRP, cholesterol, and TG Negative: HDL	
Harte et al. [20]	Pearson's correlation analysis	**All subjects:** Positive with TG (r = 0.303)	

(Continued)

Table 3 (Continuation)

Author, year	Type of analyses	Main clinical variables that correlated with LPS or LBP concentrations	
		Non-diabetic subjects	**Diabetic subjects**
Hawkesworth et al. [21]	Simple linear regression analysis	**All subjects:** Positive with Log fasting glucose (β = 0.24)	
Peraneva et al. [22]	Spearman's rank correlation test	**All subjects:** Positive with CRP (*r* = 0.221)	
Jayashree et al. [23]	Pearson's correlation analysis	**All subjects:** Positive with ZO-1(*r* = 0.252), fasting plasma glucose (*r* =0.229), 2 h post glucose (r = 0.341), HbA1c (*r* = 0.334), TG (*r* = 0.353), TNF-alpha (*r* = 0.407), IL-6 (*r* = 0.542) Negative: HDL (*r* = -0.531)	
Verges et al.[24]	Multivariable linear regression and Pearson's correlation analysis	**All subjects:** VLDL-LPS was associated with HDL-LPS (r = 0.740); LDL-LPS was associated with VLDL-LPS (r= 0.464); HDL-LPS was associated with free LPS (r = 0.592) and VLDL-LPS (0.322); free LPS was associated with HDL-LPS (r = 0.819).	
Al-Disi et al. [25]	Spearman bivariate correlations	**All subjects:** Positive with LDL-c at 3h after a high-fat meal (R=0.38) **Overweight/obese subjects:** Positive with TG (R = 0.63) and total cholesterol (R = 0.71) at baseline.	**T2DM subjects:** Positive with TG at 3 and 4 h postprandial (R= 0.52 and 0.50, respectively)

* Study that evaluated only LBP concentrations. ** LPS activity measured by LPS/HDL ratio

BPI, Bacteridal/ Permeability-increasing Protein; BMI, Body Mass Index; CRP, C- Reactive Protein; HbA1c, Glycated Hemoglobin; HDL, High-density Lipoprotein; HOMA, Homeostasis Model Assessment-Estimated Insulin Resistance; IgAGN, IgA Glomerulonephritis; LBP, Lipopolysaccharide binding protein; LDL, Low-density Lipoprotein; LPS, lipopolysaccharide; RSG, rosiglitazone; TG, triglycerides; TLR4, Toll-like receptor 4; TNF-alpha, Tumor Necrosis Factor alpha; VLDL, Very-low-density Lipoprotein.

4. Discussion

4.1. Key findings

To our knowledge, no other systematic review has assessed the association between endotoxemia and diabetes. In general, we observed that diabetic subjects presented higher fasting and postprandial LPS concentrations compared to lean non-diabetic subjects and/or obese subjects. **Figure 2** shows the possible mechanisms explaining plasma LPS increase in diabetic subjects. Fasting endotoxin concentrations seem to change with nutritional and metabolic status (healthy, obese, diabetic, etc.) [14-23, 25]. LPS was more elevated in diabetic patients with advanced complications, such as macroalbuminuria [17], and those treated with insulin [15]. These results suggest that metabolic disorder exacerbates endotoxemia. There is also evidence that endotoxin is involved in the onset of T1DM, since LPS concentrations were higher at the disease onset [18, 17].

Moreover, diabetics, IGT and obese subjects showed higher increase in postprandial endotoxemia after a high-fat meal intake compared to healthy subjects, indicating an exacerbated metabolic response [20, 25]. Harte et al. [20] emphasized that the intake of three daily high-fat meals (75 g of saturated fat) could result in constant elevated endotoxin concentrations, as each fat meal can increase plasma LPS concentrations for up to four hours.

Intestinal LPS can reach the bloodstream by two main pathways: direct diffusion due to increased intestinal paracellular permeability or by uptake and incorporation of LPS to chylomicrons (chylomicron-driven transport of LPS) [27]. Both pathways seem to contribute to increased plasma LPS concentrations in diabetic patients, since diabetics seem to have increased intestinal permeability [28] and higher LPS absorption after a high-fat meal [20, 25]. Once in the blood circulation, LPS binds to the CD14/TLR4 receptor present on the macrophages and so induces the production of proinflammatory cytokines and impairs pancreatic β-cell function [27, 28].

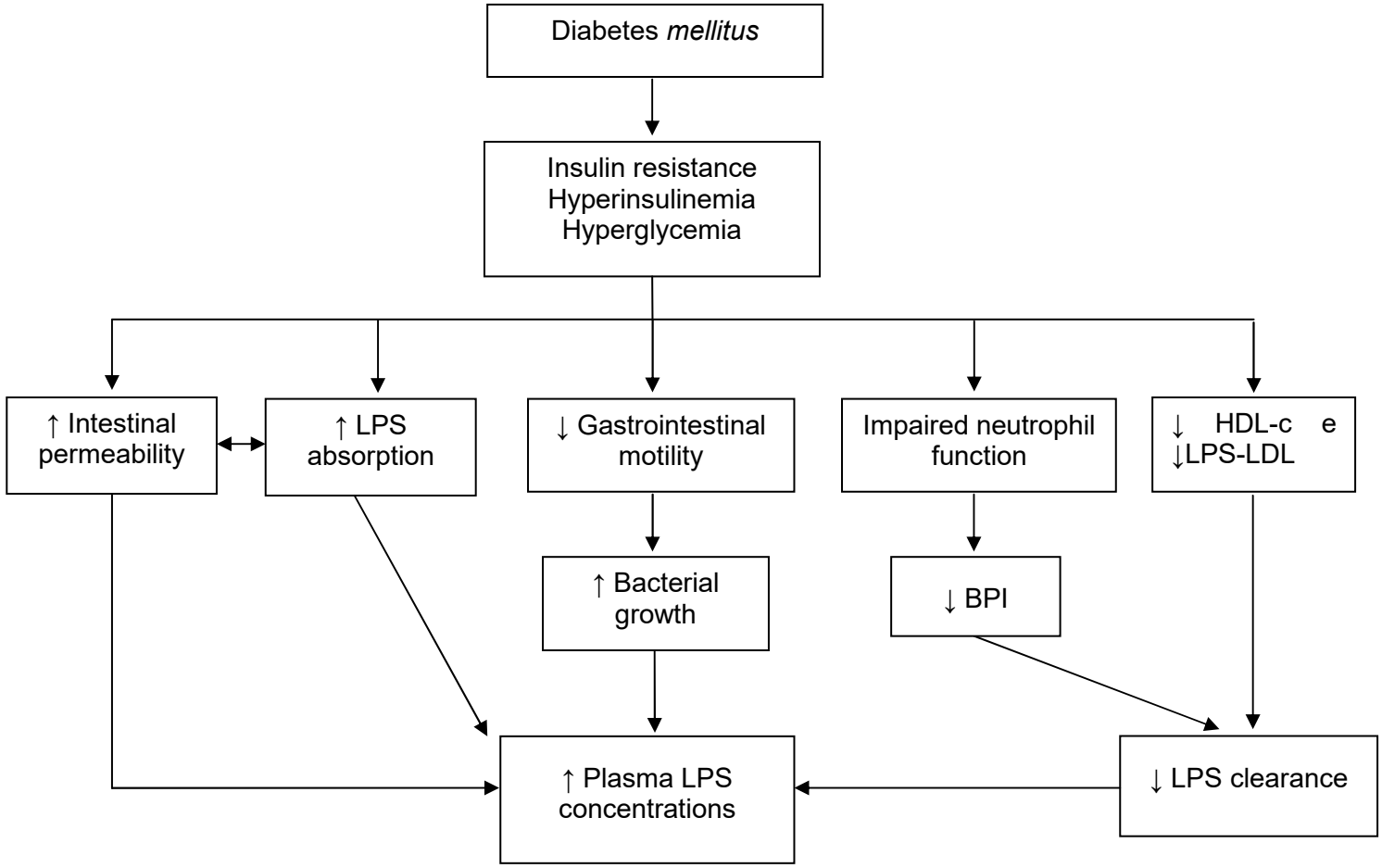

Figure 2 – Possible mechanisms explaining high-LPS concentrations in diabetic subjects.

LPS, lipopolysaccharide; BPI, bactericidal/ permeability-increasing pr otein; HDL-c, high-density lipoprotein cholesterol

Bacteria or bacterial components are effectively cleared from circulation via reticuloendothelial system. Kupffer cells and specialized macrophages in the liver recognize and remove bacterial products from circulation [22]. Hyperinsulinemia and IR impair the functionality of polymorphonuclear neutrophils and Kupffer cells [14, 29]. Similarly, hyperglycemia affects the functions of macrophages and other monocytes, suppressing the bactericidal activity of leukocytes [30, 31]. Therefore, due to hyperglycemia and hyperinsulinemia, diabetic subjects seem to have lower clearance of LPS and consequently, increased LPS concentrations.

Additionally, hypertriglyceridemia, hyperglycemia and hyperinsulinemia, commonly observed in DM, are indirect sources of endotoxemia because individuals with these problems are more susceptible to developing infections, reducing jejunum motility and increasing gastrointestinal transit time, favoring bacterial overgrowth in the small intestine and increasing gut permeability [15, 32]. Therefore, these conditions may explain not only the higher endotoxemia in diabetic patients, but also the positive correlations between LPS, triglycerides, insulin and glucose concentrations [14, 15, 17-21, 23, 25]. However, these correlations were not observed in all studies included in this review, as some authors mentioned the correlation of LPS with one or two of these variables (triglycerides, glucose and insulin) [14, 21, 23, 25], while others did not mention and/or did not assess the correlation between plasma LPS and such variables [16, 19, 20, 22, 24]. One possible explanation for these differences in correlation is the fact that the antidiabetic medication varied greatly among the studies [14-17, 19-23, 25]. In general, the antidiabetic drugs reduce insulin concentrations, improve insulin sensitivity, increase BPI and HDL-c concentrations, leading to lower plasma LPS concentrations [6, 14, 15]. The reduction of insulinemia and increase of insulin sensitivity enhance the functionality of neutrophils and increase the clearance of LPS [6, 29]. BPI reduces LPS-activated immune response, since it reduces the binding of LPS to LBP [6]. HDL favors LPS detoxification [24, 33]. Therefore, the antidiabetic medication seems to reduce the pro-inflammatory effects of LPS. RSG had greater effect on endotoxemia compared with other antidiabetic medication,

which may partially explain the anti-inflammatory effects of this drug [15]. Another difference is that some authors used regression models to assess their data [15, 17, 21, 24], which is considered more robust than other methods, such as simple correlations, used to do this type of analyses [34, 35]. Using these models, one can study several independent variables, their relationships and the effects they have on dependent variables [34, 35].

After the consumption of a high-fat meal, the insoluble fraction of LPS (lipid A) is incorporated into the micelles and absorbed with chylomicrons [32, 36]. The binding of LPS to lipoproteins seems to inhibit endotoxin activity, and this ability seems to be dependent on the composition of the lipoproteins. Human reconstituted HDL, containing purified apoprotein A-I (apoA-I), phosphatidylcholine and cholesterol, neutralizes endotoxin in the blood more effectively than other lipoproteins [33]. Verges et al. [24] examined the metabolism of LPS in different lipoproteins and suggested a catabolic pathway for LPS. After reaching the liver and being removed from chylomicrons, free LPS transfers first to HDL, and then to VLDL [24]. The LPS-bound LDL fraction (LDL-LPS) seems to be mainly derived from VLDL catabolism. Diabetic patients had lower LDL-LPS concentrations due to reduced VLDL catabolism, which may represent an impaired catabolic pathway [24]. Furthermore, it is common for diabetics to present reduced HDL concentrations [17], which contributes to reduce LPS clearance and increase the inflammatory status, exacerbated by high concentrations of endotoxin in diabetic subjects [20]. Serum LPS activity (measured by LPS/HDL ratio) seems to strongly correlate with metabolic syndrome components, such as triglycerides, fasting glucose, and HDL concentrations [17, 19]. Therefore, high serum LPS activity combined with common metabolic abnormalities in DM may contribute to the development of macrovascular and microvascular complications, and so it is a potential tool to assess the metabolic risk profile in diabetic patients [17, 19].

In summary, DM and its metabolic abnormalities characterized by insulin resistance, hyperinsulinemia, and hyperglycemia, lead to increased intestinal permeability and higher LPS absorption, increasing plasma LPS concentrations. Concomitantly, reduced intestinal motility commonly observed in patients with DM, favors bacterial growth, exacerbating intestinal integrity breakdown and

increasing endotoxemia. Furthermore, reduced HDL-c and impaired neutrophil function, also common situations in DM, lead to reduced BPI concentrations and consequently lower LPS clearance. Altogether, these mechanisms seem to negatively affect endotoxemia, which may worsen DM control.

4.2. Limitations

Due to the observational nature of most of the studies included in this review, they describe only associations and not causalities. As the study of subclinical endotexemia is relatively recent, several articles did not meet the inclusion criteria we adopted. Furthermore, the heterogeneity of the articles and the different units of measurement of the variables used to assess endotoxemia did not allow us to perform a meta-analysis. Our findings also indicate that there was a considerable variability in quality and risk of bias among the studies included. That is another reason why we did not conduct a meta-analysis and the reason why it was difficult to make strong inferences from the results obtained in the included studies. However, regardless of these limitations these findings highlight the importance of LPS metabolism in patients with diabetes. Therefore, the analyzed data allowed us to propose possible mechanisms that could be investigated in future research.

Although LAL test is widely used to assess endotoxemia, this test is not capable of discriminating "toxic LPS" (diphosphoryl) and "nontoxic" (monophosphoryl) LPS [37] and its use has not been approved for clinical use [38]. Another disadvantage of this test is its indeterminate interlaboratory variability [38], as noted by the very different LPS concentrations among the articles included in this review. Such limitations to assessing plasma LPS in humans tend to hinder the establishment of the true relationship between clinical variables. However, there is no “gold-standard” test recommended to assess endotoxemia.

5. Conclusion

T1DM and T2DM seem to increase metabolic endotoxemia. Hyperglycemia and hyperinsulinemia cause increased intestinal permeability, decreased functionality of neutrophils and antimicrobial factors such as BPI, as

well as impaired LPS catabolic pathway mediated by lipoproteins, leading to a lower LPS clearance and higher concentrations of circulating endotoxin in diabetic patients.

This systematic review reveals what is known to date about the influence of endotoxemia on DM. This evidence is novel and it suggests that elevated LPS concentrations could be an important factor affecting glucose metabolism and could be implicated in complications associated with DM. Thus, specific strategies for modifying endotoxemia could be useful for treating DM. Future research on this topic must be well designed to reduce bias risk and should infer causality. Double-blind, randomized, controlled trials that assess the effect of changing LPS concentrations (by dietary or medication modifications, for example) on glucose homeostasis will hopefully help address these issues. Future studies should also elucidate the complex mechanisms related to the action of LPS on diabetes, since DM is a multifactorial disease and various confounders such as diet, age, medication, smoking and obesity influence both DM and endotoxemia. Thus, a better understanding of the interaction of these factors is still needed.

6. Acknowledgments

We thank Instituto Federal do Sudeste de Minas (IF Sudeste MG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors' contributions were as follows: JMGG and JAC contributed to the study conception and design, performed the literature search, analyzed the data, and wrote the paper. RCGA contributed to the interpretation of data, wrote and edited the manuscript. All authors read and approved the final manuscript.

7. Conflict of Interests/Financial Disclosure

None for all authors.

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3. OBJECTIVES

3.1. General objective

To evaluate the effects of fat-free milk consumption on weight loss, body composition, metabolic syndrome (MetS), and cardiometabolic outcomes in adults with type 2 diabetes *mellitus*.

3.2. Specific objectives

To evaluate the effects of fat-free milk consumption on:

- Obesity (body weight, body mass index (BMI), fat mass (FM), fat-free mass (FFM), waist circumference (WC) and waist-hip ratio (WHR)) and glycemic control (serum uric acid, fructosamine, glycated hemoglobin (HbA1c), insulin, and fasting glucose concentrations, HOMA2-IR) (**Article 1,** a crossover randomized clinical trial);
- MetS components and cardiometabolic outcomes (WC, serum fasting glucose, triglycerides (TG), blood pressure (BP), total cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), HDL/LDL ratio, HDL/TG ratio and lipid accumulation product (LAP) index) (**Article 2**, a crossover randomized clinical trial);
- Body weight, body composition (BMI, WC, WHR, FM, FFM), and IR (serum fasting glucose, fructosamine, HbA1c, and insulin concentrations, HOMA-IR, HOMA2-IR, and TyG index) (**Article 3**, a parallel group randomized clinical trial).

4. PARTICIPANTS AND METHODS

This thesis contains two crossover studies (Articles 1 and 2) and a parallel design study (Article 3). Other results of this project were presented in the thesis authored by Jorge Assis Costa, entitled “Impacto do aumento da ingestão de cálcio lácteo associado a atividades educativas no controle metabólico em diabéticos tipo 2” (Costa, 2016).

This study was conducted according to the Declaration of Helsinki guidelines and all procedures involving human participants were approved by the Committee of Ethics in Human Research of the Federal University of Viçosa/Brazil. Written informed consent was obtained from all subjects. The present trial was registered at *www.clinicaltrials.gov*, as “Dietary Calcium Supplementation, Gut Permeability and Microbiota in Type 2 Diabetics” (ID no. NCT02377076).

4.1. Participants

Study participants were recruited through public advertisements in the town of Viçosa, Minas Gerais, Brazil. Recruitment was initiated on February 2, 2014, and ended on June 3, 2015 when the required number of subjects for the study was obtained. Eligible subjects were adults of both genders with T2DM treated with only diet or with diet plus oral hypoglycemic agents, that had metabolic syndrome (MetS) (Alberti et al., 2009), had low habitual Ca intake (< 600 mg/d), were between 20 and 59 years of age, had a dietary restraint<14 (Strunkard & Messic, 1985), had light to moderate physical activity levels (PAL) (Pardini et al., 2001), and had T2DM for at least one year.

Exclusion criteria were the following: (1) smoking; (2) use of Ca, vitamin D, zinc (Zn) or magnesium (Mg) supplements or medication that affects the metabolism of these micronutrients; (3) use of drugs (except hypoglycemic drugs), herbs, or diets for weight loss; (4) on hormone replacement therapy; (5) menopause or post menopause; (6) recent weight gain or loss (±5 kg) over the previous three months; (7) recent change in PAL over the previous three months; (8) aversion or intolerance to the shakes provided during the study; (9) alcohol consumption of more than 12g/d for women and 24g/d for men; (10) eating disorders; (11) endocrine (except T2DM and obesity), kidney, or liver

pathology; (12) Ca malabsorption; (13) history of recurrent nephrolithiasis; (14) history of gastric surgery or current gastric disease including gastroparesis; (15) consumption of more than 350 mg/d of caffeine; (16) pregnancy or lactation; (17) anaemia; and (18) changes in medication type or dosage during the study. Sample size was calculated (Mera et al., 1998) considering 1% difference in glycated hemoglobin (HbA1C), and a statistical power of 80%.

4.2. Study design

Participants were initially randomly assigned by simple randomization procedures (computerized random numbers) to high-Ca fat-free milk phase (MD) (equivalent to ~3 fat-free milk portions) or low-Ca control phase (CD) phases in 1:1 ratio. Participants and data analysts were blinded. An energy restricted diet (restriction of 500 kcal/d) containing 800 mg of dietary Ca/d was prescribed. Subjects daily consumed in the laboratory a breakfast shake containing 700 mg (MD) (equivalent to approximately 3 servings of fat-free milk) or 6.4 mg (CD) of Ca. All other meals were consumed in free-living condition in both phases. Participants were instructed to maintain constant PAL and medication use during the study.

4.2.1. Article 1:

This is a randomized, crossover clinical trial of two 12-week phases separated by a washout period of -8-weeks. PAL, food intake, body composition (fat mass (FM) and fat-free mass (FFM)), anthropometric (body weight, WC, waist-hip ratio (WHR)), and biochemical variables (serum Ca, P, Mg, glucose, uric acid, HbA1c, vitamin D, insulin, fructosamine and parathormone (PTH) concentrations) were evaluated at baseline and after 12 weeks of each dietary experimental phase (**Figure 1, Article 1**).

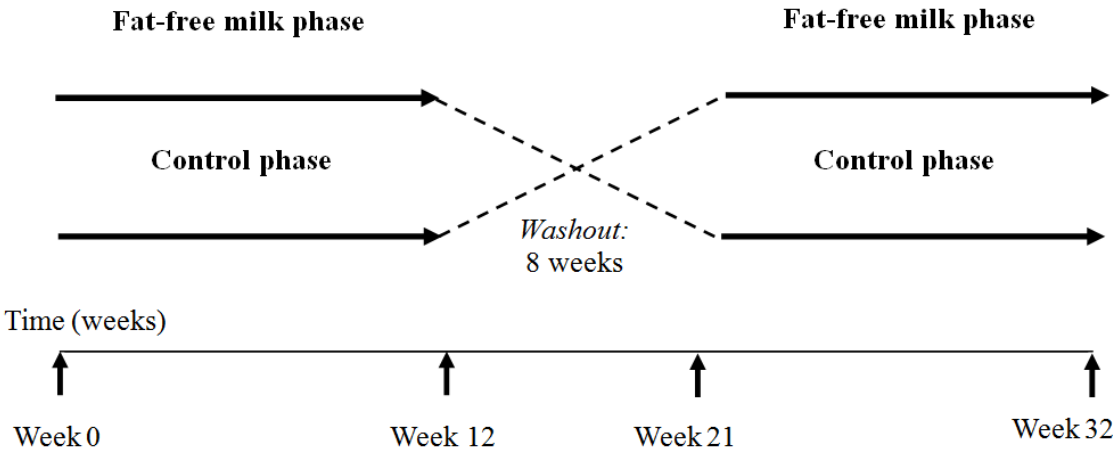

Figure 1, Article 1 – Study design. Physical activity level, food intake, body composition, anthropometric measures, and biochemical variables were assessed on the weeks 0, 12, 21 and 32 (N = 14).

4.2.2. Article 2:

This is a randomized, crossover clinical trial of two 12-week phases separated by a washout period of -8-weeks.

PAL, nutrient and energy intake, MetS components (WC, fasting glucose, fasting triglyceride (TG), HDL-c and BP) [15], other cardiometabolic risk factors (total cholesterol, LDL-c, VLDL-c, insulin, HDL/LDL ratio, HDL/TG ratio and lipid accumulation product (LAP) index)), and Ca homeostasis markers (serum total Ca, P, Mg, parathormone (PTH) and 25-hydroxivitamin D concentrations) were evaluated at baseline and after 12 weeks of each dietary phase (**Figure 1, Article 2**).

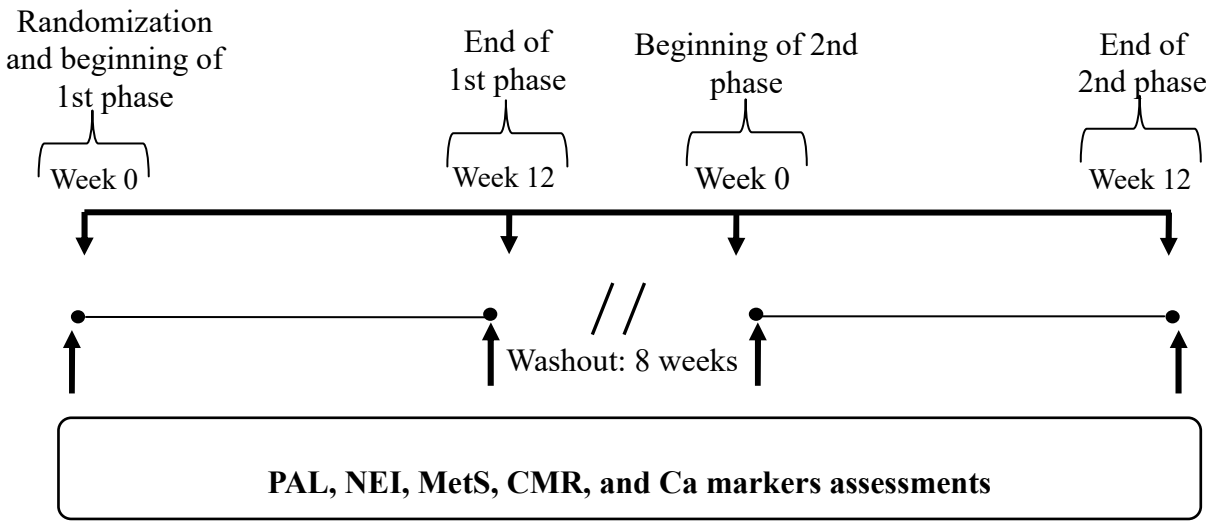

Figure 1, Article 2 – Study design. Physical activity level (PAL), nutrient and energy intake (NEI), metabolic syndrome components (MetS) cardiometabolic risk factors (CMR), and calcium homeostasis markers (Ca markers) were assessed before and after each phase (fat-free milk and control phase) (N = 14).

4.2.3. Article 3:

This is a 12-week duration, parallel group randomized clinical trial. Subjects were randomly assigned to one of the groups: to high-Ca phase (MD) (equivalent to ~3 fat-free milk portions) or low-Ca control diet (CD). Food intake, body composition (fat mass (FM) and fat-free mass (FFM)), anthropometric (body weight, WC, waist-hip ratio (WHR)), and biochemical variables (serum glucose, triglycerides (TG), HbA1c, fructosamine, insulin, HOMA-IR, HOMA2-IR, TyG) were evaluated at baseline and after 12 weeks of intervention **(Figure 1, Article 3).**

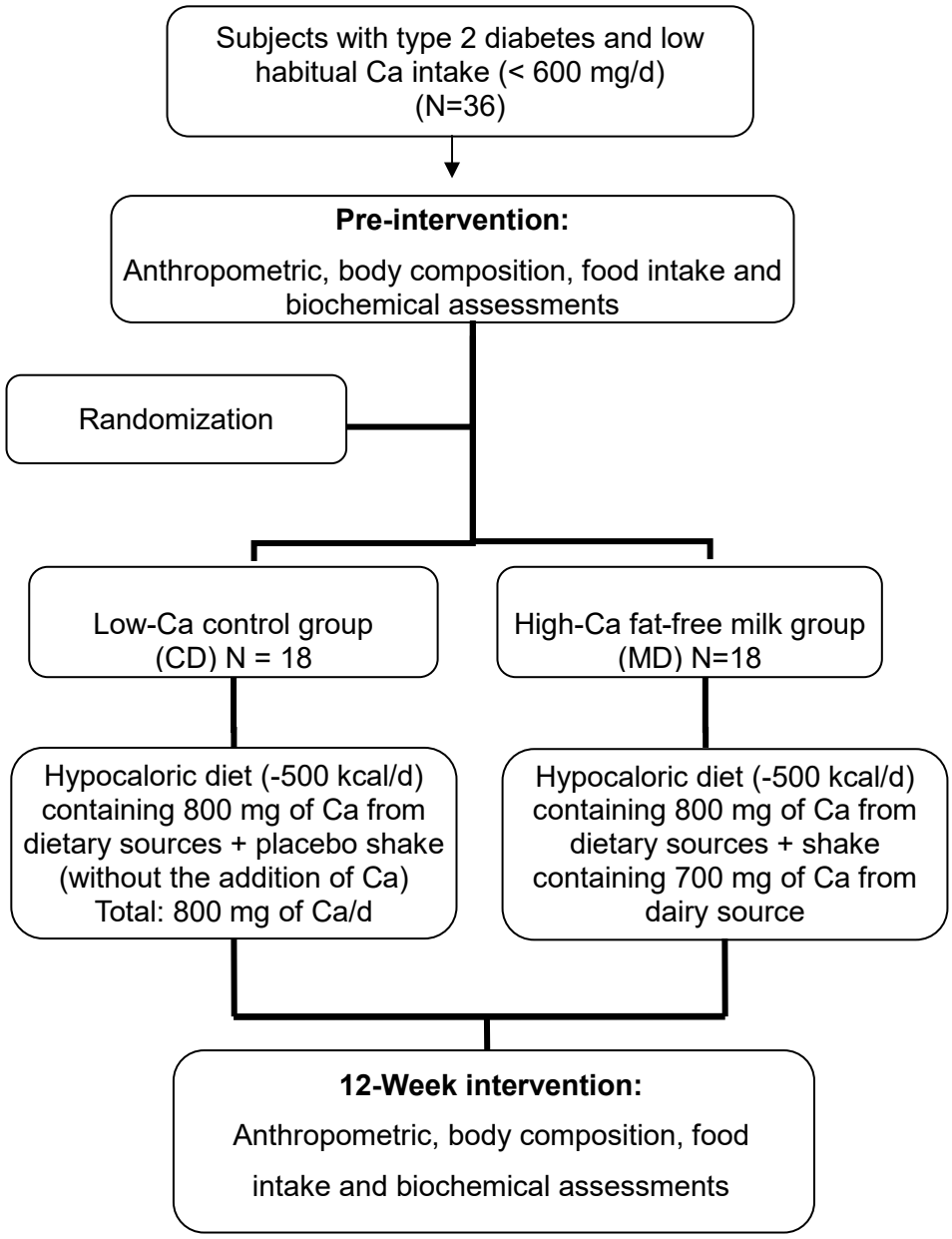

Figure 1, Article 3 – Study design. Ca: calcium.

4.3. Physical activity assessment

PAL was assessed using the long format International Physical Activity Questionnaire (IPAQ), version 6, validated for the Brazilian adult population (Pardini et al., 2001). The total amount of time spent in physical activity per week was estimated by the sum of each item (i.e., vigorous intensity, moderate intensity, and walking) obtained from IPAQ. Total weekly physical activity (MET-min week−1) was estimated by adding the products of reported time for each item by a MET value specific for each physical activity category. PALs were classified as: vigorous PAL = 8.0 METs, moderate PAL = 4.0 METs, and walking = 3.3 METs (Pardini et al., 2001). To verify possible changes in PAL, IPAQ was applied at baseline and at end of each phase (CD and MD).

4.4. Food intake assessment

Habitual Ca consumption was assessed at baseline using a quantitative food frequency questionnaire (QFFQ) (Ribeiro & Cardoso, 2002). Food intake at baseline and after 12 weeks of each experimental phase was assessed using three non-consecutive days (two weekdays and a weekend day) food records. Participants were trained to keep free-feeding dietary records at baseline to increase data reliability. Each dietary record was reviewed with the participants to ensure accuracy and completeness. Moreover, a food porting photo album was used to improve data quality (Monteiro et al., 2007). The amounts of foods registered in household measures were converted into grams for energy intake, macronutrients, Ca, P, Mg, Zn, and dietary fiber intake analyses using DietPro, version 5.1i (July, 2015). A single dietitian analyzed the food records. The revised Goldberg method was used to categorize misreported reported energy intake (rEI) (Black, 2000). Participants were classified as underreporters, acceptable reporters, or overreporters using the ratio of rEI to total energy expenditure (TEE). TEE was calculated from the product of basal metabolic rate (FAO/WHO/UNU, 1985) and PAL. PAL was assumed to be 1.55 (light activity) for all subjects (FAO/WHO/UNU, 1985). A 95% confidence interval (CI) was calculated, and individuals who fall outside of the CI 95% were classified as under or overreporters and were excluded from statistical analysis.

4.5. Dietary intervention

Each participant's daily energy requirement was based on the Estimated Energy Requirement (EER) (Trumbo et al., 2002). Then, 500 kcal/d were subtracted for dietary restriction. Diets were prescribed according to the American Diabetes Association nutrition recommendations (ADA, 2016) and considering the nutritional composition of the breakfast shakes provided during the study. MD and CD prescribed diets presented similar contents of macronutrients, vitamin D, P, Mg, Zn, and dietary fiber. MD prescribed diet contained 1,500 mg and CD had 800 mg of Ca/d. Participants were given meal patterns and one list discriminating the types of foods to help them in their food choices. The foods were grouped in that list considering their energy and Ca content. Participants received individualized nutritional counseling at every 2 weeks to increase prescribed diet adherence. An experienced dietitian assessed eating patterns and habitual food intake, exercise and medication patterns. Nutrition counseling was provided to stimulate healthy eating habits, including adequate consumption of dietary fiber, water; besides avoiding alcohol consumption, etc. according to ADA recommendations (ADA, 2016). Energy and nutrient requirements were adjusted according to the nutritional requirement of each subject right before the beginning of the second experimental phase. In the washout period, participants were told to maintain their normal diet, which was assessed through three non-consecutive days (two weekdays and a weekend day) food records.

4.6. Breakfast shakes

Twelve shake types (six for MD and six for CD experimental phase) were developed for consumption at breakfast. Shakes flavors (frozen fruit pulps or chocolate powder) were the same for both phases. They presented similar macronutrient, vitamin D, sodium, and dietary fiber contents, differing mainly in their Ca content (**Table 1**). Shakes were also visually very similar, ensuring participants remained unaware of phase differences and the purpose of the intervention. High-Ca shakes contained fat-free milk powder (Itambé® enriched with iron, vitamins A, C, and D, and Ca) reconstituted in water (250 mL). To

ensure similarity to high-Ca shakes, low-Ca shakes contained whey protein (BemVital®, Diacom), sucrose, sodium chloride (Cisne®), and a powder supplement containing iron (iron chelate) and vitamins A (retinol acetate), C (ascorbic acid), and D3 (cholecalciferol). That supplement was prepared by a certified compounding pharmacy. Shakes were prepared mixing all the ingredients in a blender right before ingestion. Breakfast shakes flavors were offered in random order, according with study phase.

Subjects daily consumed a shake in the laboratory for 84 consecutive days (12 weeks) in each phase. In case any subject eventually could not come to the laboratory, the shake was consumed in their homes/ jobs.

Table 1 – Ingredients and nutrient composition of the breakfast meals according to study phases [a]

Ingredients (1 serving of 500 ml)	**Study phase**	
	MD	**CD**
Fat-free milk powder (g)	47.0	0.0
Sugar (g)	0.0	23.5
Whey protein (g)	0.0	18.02
Sodium chloride (g)	0.0	0.7
Micronutrient supplement (mg)[b]	0.0	4.0
Frozen fruit pulps or chocolate powder (g)[c]	100 or 10	100 or 10
Water (ml)	250	250
Nutrient composition (mean ± SD)		
Energy (kcal)	197.8 ± 11.1	198.0 ± 11.1
Carbohydrate (g)	31.9 ± 2.9	31.9 ± 2.9
Fiber (g)	1.9 ± 2.2	1.9 ± 2.2
Protein (g)	17.1 ± 0.2	17.1 ± 0.2
Fat (g)	0.1 ± 0.3	0.1 ± 0.3
Calcium (mg)	710.5 ± 3.7	6.4 ± 3.7***
Iron (mg)	10.0 ± 0.1	10.1 ± 0.1
Phosphorus (mg)	799.5 ± 7.8	13.7 ± 7.4***
Mg (mg)	58.9 ± 3.9	7.8 ± 3.9***
Sodium (mg)	280.9 ± 3.3	280.9 ± 3.3
Zinc (mg)	1.91 ± 0.1	0.12 ± 0.1***
Vitamin A (mcg)	423.0 ± 0.0	423.0 ± 0.0
Vitamin C (mg)	163.8 ± 243.3	163.8 ± 243.3
Vitamin D (mcg)	3.5 ± 0.0	3.5 ± 0.0

MD, High-calcium fat-free milk diet; CD, Low-calcium control diet. [a] Calculated by DietPro® software or according to food labels. [b] Micronutrient powder supplement containing iron (iron chelate) and vitamins A (retinol acetate), C (ascorbic acid), and D3 (cholecalciferol) prepared by a certified compounding pharmacy. [c] Shakes presented similar flavors (frozen fruit pulps or chocolate powder), total of six different flavors. *** $P<0.001$ by Student's t test.

4.7. Anthropometric and body composition measurements

Participants fasted overnight. All measurements were assessed while the participants were barefoot and wearing light clothing. Participants abstained from strenuous exercise, as well as caffeine and alcohol consumption 48 hours prior to these assessments. Women were not in the menstrual period.

Body weight was assessed using an electronic platform scale (Model 2096 PP, Toledo, Brazil) with a capacity for 150 kg and precision of 50 g. Height was measured using a stadiometer with a scale of 0 to 220 cm, precision 0.1 cm (SECA 206,Seca). Both measurements were performed according to Jellife (1968). Body mass index (BMI) was calculated from the ratio of weight (kg) to height squared (m^2). WC and hip circumference (HipC) were measured using a flexible inelastic tape. WC was measured at the midpoint between the lowest rib and the iliac crest with a precision of 0.1 cm (Wang et al., 2003), and HipC was measured at the largest circumference between the anterior iliac crest and the greater trochanter (WHO, 2011). WHR was calculated by dividing WC by HipC.

Body composition was assessed using a Prodigy densitometer (GE Lunar Medical Systems, Milwaukee, WI, USA). The scanner was calibrated daily against the standard calibration block supplied by the manufacturer to control for possible baseline drift. The participants lay supine on the bed and were scanned from head to toe. The scanner uses a narrow fan beam (4.5°) parallel to the longitudinal axis of the body. Scans were analyzed using EnCoreTM, version 13.5. The manufacturer's algorithms provide a three-compartment analysis consisting of non-bone lean tissue mass, FM, and bone mineral content (BMC) ash. FFM was defined as the sum of lean tissue mass and BMC. Data from BMC were not shown. In our laboratory, the within coefficients of variation for the measurement of percentage fat mass (%FM) and percentage fat-free mass (%FFM) in five participants, measured twice (with repositioning), were 1.9% and 2.0 %, respectively (data not shown).

4.8. Biochemical assays

Venous blood samples were obtained after 12 hours of overnight fasting. Serum glucose levels were measured by enzymatic colorimetric assay (BS200,

Mindray, Diagnostic Laboratory Instrument Inc., Shenzhen, China). The HbA1c was measured by an ion-exchange High Performance Liquid Chromatography (HPLC). Serum fructosamine levels were measured by a colorimetric nitroblue tetrazolium assay (Technicon Co., Japan). Serum uric acid, P, and Mg levels were measured using a colorimetric assay (Bioclin kit, Quibasa Basic Chemical Ltda, Belo Horizonte, Brazil). Serum PTH and insulin levels were measured with an electrochemiluminescence immunoassay (Elecsys Modular-E-170, Roche Diagnostics Systems). Serum 25-hydroxivitamin D levels were determined by chemiluminescent microparticle immunoassay (Architect i2000, Abbott Diagnostics). Serum total Ca levels were assessed by Ca arsenazo III method (Mira Plus, Roche Diagnostic Systems).

IR was calculated using the updated homeostatic model assessment of insulin resistance (HOMA2-IR) index, which considers a more accurate physiological basis for the prediction of homeostatic response than did the HOMA-IR index. This index estimates the functions of beta cells (% B) and insulin sensitivity (% S). The HOMA2-IR index was obtained using HOMA Calculator, version 2.2.2, available at *https://www.dtu.ox.ac.uk/homacalculator/*.

In the Article 3, IR was assessed by:

1. HOMA-IR (*Homeostasis Model Assessment of Insulin Resistance*), calculated according to the formula:
   Glycemia (mMol) . Insulin (uU.mL^{-1}) / 22,5 (Matthews et al., 1985);
2. HOMA2-IR, calculated by *HOMA Calculator* software, available at: *https://www.dtu.ox.ac.uk/homacalculator/;*
3. TyG, a product of fasting glucose and serum triglycerides, calculated according to the formula:
   Ln triglyceridemia (mg.dL^{-1}) . glycemia (mg.dL^{-1})/2 (Simental-Mendía, Rodríguez-Morán, & Guerrero-Romero, 2008).

4.9. MetS components and cardiometabolic measures

MetS components assessed were WC, fasting glucose, fasting TG, HDL-c, and BP, using the 'harmonized' version of the MetS (Alberti et al., 2009). Other cardiometabolic risk factors assessed were total cholesterol, LDL-c, VLDL-c, insulin, HDL/LDL ratio, HDL/TG ratio, and LAP index.

WC and venous blood samples were obtained after 12 hours of overnight fasting. WC was measured using a flexible inelastic tape at the midpoint between the lowest rib and the iliac crest with a precision of 0.1 cm (Wang et al., 2003). Serum glucose, TG, total cholesterol, HDL-c, LDL-c and VLDL-c concentrations were measured by enzymatic colorimetric assay (BS200, Mindray, Diagnostic Laboratory Instrument Inc., Shenzhen, China). Insulin concentrations were measured with an electrochemiluminescence immunoassay (Elecsys Modular-E-170, Roche Diagnostics Systems). Systolic (SBP) and diastolic BP (DBP) were measured using a calibrated automated sphygmomanometer (Omron HEM-742INT IntelliSense®), after a resting period of at least 10 min in the sitting position. An appropriate arm cuff was used. Arm position was adjusted so that the cuff was at the level of the right atrium. BP was measured on the dominant arm, every 3 min for 15 min, at the constant temperature of 22–24ºC. The first value was discarded, and the mean of the last four readings was used in the analysis. HDL/LDL ratio was calculated by dividing HDL-c by LDL-c values. HDL/TG ratio was calculated by dividing HDL-c by TG concentrations. The LAP index was calculated as (WC [cm] − 65) × (TG concentration [mM]) for men, and (WC [cm] − 58) × (TG concentration [mM]) for women (Kahn, 2005).

4.10. Calcium Homeostasis Markers

Serum total Ca concentrations were assessed by Ca arsenazo III method (Mira Plus, Roche Diagnostic Systems). Serum P and Mg concentrations were measured using a colorimetric assay (Bioclin kit, Quibasa Basic Chemical Ltda, Belo Horizonte, Brazil). Serum PTH concentrations were measured with an electrochemiluminescence immunoassay (Elecsys Modular-E-170, Roche Diagnostics Systems). Serum 25-hydroxivitamin D levels were determined by chemiluminescent microparticle immunoassay (Architect i2000, Abbott Diagnostics).

4.11. Statistical analysis

4.11.1. Articles 1 and 2

Statistical analyses were conducted using the Statistical Package for

Social Sciences for Windows, version 20.0 (IBM). All variables were examined for normality of distribution according to the Shapiro-Wilk test at 5% significance. Data are expressed as means and standard deviations, unless otherwise indicated. An independent-samples t test was conducted to identify possible differences between who started with either MD (n = 7) or CD (n = 7) at baseline. To verify the efficacy of the washout period, a paired t test was conducted between the initial baseline data and the post-washout baseline data. Baseline data were calculated before each intervention period. Once the efficacy of the washout period was verified, the initial baseline and post-washout baseline data were combined and the final data from each study arm (n = 14 each for the 2 phases) were combined. Then, data within sessions were analyzed using the paired t-test or Wilcoxon rank sum test, pairing results from the same individual before (baseline) and after (12 weeks) each dietary intervention (CD or MD phase), considering *P* values less than or equal to 0.05 as significant. Data on changes from the baseline over the 12 weeks of the intervention (deltas, i.e., the final value minus the baseline value) were compared between the sessions using the paired t-test or Wilcoxon rank sum test, with Bonferroni correction for multiple comparisons. The criterion of significance was $P < 0.025$, two tailed.

4.11.2. Article 3

Statistical analyses were conducted using the Statistical Package for Social Sciences for Windows, version 20.0 (IBM). All variables were examined for normality of distribution according to the Shapiro-Wilk test at 5% significance. Data are expressed as means and standard deviations, unless otherwise indicated. Bartlett test, at 1% significance, was applied to assess the homogeneity of the residual variances. Data within groups were analysed using paired t-test or Wilcoxon rank sum test, pairing results from the same individual before (baseline) and after 12 weeks of intervention (CD or MD group), considering *P* values ≤ 0.05 as significant. Data on changes from the baseline over the 12 weeks of the intervention (deltas, i.e., the final value minus the baseline value) were compared between the groups using the t -test or Mann-Whitney test, with Bonferroni correction for multiple comparisons. The criterion

of significance was P< 0.025, two tailed.

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5. RESULTS

5.1. ARTICLE 1

Fat-free milk enhances weight loss, improves body composition, and promotes glycemic control in adults with type 2 diabetes *mellitus* – a randomized clinical trial

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Keywords: Body composition; Calcium; Dairy; Diabetes; Obesity

Abstract: 278 words

Main text: 4522 words

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Abstract

Background

Dietary calcium (Ca) and fat-free dairy have been considered potential regulators of body weight and glycaemia. However, there is no consensus among authors about that, and only a few clinical trials have examined Ca's effect in adults with type 2 diabetes.

Objective

We evaluated the effects of high-Ca fat-free milk versus low-Ca control diet on adiposity and on glycemic control.

Design

Fourteen subjects with type 2 diabetes (aged 49.5 (SD 8.6) years, BMI 29.4 (SD4.5) kg/m^2, low habitual Ca consumption (< 600 mg/d)) were included in this randomized, crossover clinical trial. Subjects participated of two 12-week experimental phases (high-Ca fat-free milk phase (MD) or low-Ca control phase (CD)) separated by 8-week washout. Subjects daily consumed in the laboratory a breakfast shake containing 700 mg (MD) or 6.4 mg (CD) of Ca. Energy restricted diets containing 800 mg of dietary Ca/d were prescribed.

Results

Dietary records data indicated the consumption of 1,200 mg of Ca/d during MD and of 525 mg of Ca/d during CD. There was a greater reduction in body weight, body fat mass, waist circumference, and waist-hip ratio after MD. Serum 25-hydoxyvitamin D and HOMA2-B% increased and serum uric acid, parathormone (PTH), and glycated hemoglobin (HbA1c) levels reduced after MD. Additionally, changes from baseline in terms of serum uric acid, glucose, HbA1c, and PTH concentrations were lower and those of HOMA2-B%, serum Ca, and 25-hydoxyvitamin D were higher after the MD than after CD.

Conclusion

The consumption of approximately 3 servings of fat-free milk and 1,200 mg of dietary Ca/d enhanced weight loss, improved body composition, and promoted glycemic control in subjects with type 2 diabetes and low habitual Ca consumption (<600 mg/d).

Trial registration: ClinicalTrials with nr NCT02377076

1. Introduction

The high prevalence and incidence of type 2 diabetes mellitus (T2DM) have posed a challenge for public health because of the resulting metabolic complications and high cost associated with the disease (ADA, 2016). Obesity is a major factor that favors insulin resistance (IR), thereby increasing the risk of T2DM and impaired glycemic control (ADA, 2016; WHO, 2003). Although dietary intervention has been recognized as useful in the treatment of T2DM, current guidelines emphasize the roles of macronutrients in glycemic control in T2DM patients, giving little attention to micronutrients (ADA, 2016). The benefits of dietary calcium (Ca) and dairy on T2DM control have been suggested (Pittas et al., 2007; IOM, 2011; Pasin and Comerford, 2015). However, Dietary Reference Intakes (DRIs) for Ca only considers its benefits in bone health (IOM, 2011).

Ca consumption is inadequate in many parts of the world (Imamura et al., 2015). An adequate Ca consumption seems to improve glucose homeostasis in T2DM adults, particularly among low habitual consumers (<600 mg/d) (Pittas et al., 2007). That effect seems to be mainly due to the consumption of fat-free dairy (Candido et al., 2013) associated with energy restricted diets (Abargouei et al., 2012). Possible mechanisms involving low-fat dairy components that may favor body weight and glycemic control are still not well understood. However, Ca, vitamin D, dairy proteins, zinc (Zn), and magnesium (Mg) may act on satiety, appetite regulation, fat oxidation, besides insulin production and release (Pittas et al., 2007; Acheson et al., 2011; Yahya et al., 2011; Candido and Bressan; 2014). The role of whey protein in T2DM has been better established than casein. Whey protein affects glycemic control by direct mechanisms (i.e., improving insulin secretion and insulin response), and indirect mechanisms (i.e., improving fat loss, lean-mass retention and satiety) (Pasin and Comerford, 2015).

Recently, a meta-analysis of randomized clinical trials indicated that increasing dairy intake resulted in greater body weight and body fat loss, while lean mass loss was attenuated in subjects on weight loss diets (Stonehouse et al., 2016). However, the effect of different dairy sources could not be distinguished (e.g., milk vs. cheese vs. yoghurt) (Stonehouse et al., 2016).

Since dairy sources have different nutritional composition, especially with respect to microorganisms, it is important to investigate if milk, which is relatively inexpensive and affordable food, influences obesity control.

The effects of dairy components on body weight and body composition in athletes and healthy overweight and obese adults have been extensively investigated (Zemel et al., 2004; Bowen et al., 2005; Gunther et al., 2005; Harvey-Berino et al., 2005; Thompson et al., 2005; Zemel et al., 2005; Zemel et al., 2009; Stancliffe et al.,2011; Josse and Philips, 2012; Jones et al., 2013; Torres and Sanjuliani, 2013; Devries and Philips, 2015). However, few studies have examined dairy's effect in individuals with T2DM (Pittas et al., 2007; Pasin and Comerford, 2015). Clinical evidences described in a systematic review indicated that dairy foods and dairy proteins (mainly whey protein) consumption might improve insulin secretion in T2DM adults (Pasin and Comerford, 2015). However, due to the different doses consumed, different dairy sources, and the short-term nature of the clinical trials it is difficult for us to make effective dietary recommendations based on results obtained in the studies included in that review (Pasin and Comerford, 2015).

Considering the relevance of glycemic control to prevent T2DM complications and the scarcity of long-term studies concerning the role of dairy and Ca consumption in T2DM, we evaluated the effects of fat-free milk consumption on obesity and on glycemic control in subjects with T2DM.

2. Materials and Methods

Described on pages 70 - 84, "PARTICIPANTS AND METHODS".

3. Results

Four men and 10 women completed the study (49.5 (SD of 8.6) years old, and BMI of 29.4 (SD of 4.5) kg/m^2). From a total of 68 potential participants contacted, most of them (52) did not fully meet the inclusion/exclusion criteria adopted in the study. Therefore, 16 participants were initially included. However, 2 did not complete the study due to personal reasons. A total of 14 participants completed the study protocol. The present study had a power of 80% to detect a reduction of 0.7% in HbA1c, considering the standard deviation of 1.4% in that

variable (post-hoc power).

3.1. Food intake

Ca, P, Mg, and fiber intake increased after MD and remained unchanged after CD (**Table 2**). The Ca:P ratio was 0.49 and 0.66 at baseline, and 0.74 and 0.62 post-treatment for the MD and CD, respectively. Dietary fiber, Ca and P intake increased after MD compared to CD (**Table 3**). According to the revised Goldberg method [30], only 50% (n=7) and 42.9% (n=6) of participants in MD and CD phase, respectively, were categorized as acceptable energy intake reporters at baseline and after 12-week intervention. Participants were not compliant to the energy restriction prescribed (- 500 kcal/d) (**Table 3**).

3.2. Anthropometry and body composition

All the anthropometric variables and FM reduced after the MD phase, but did not change after the CD phase compared to baseline (**Table 2**). MD promoted a greater reduction in body weight, FM, WC and WHR (**Figure 2**). FM% and WC decreased in MD, while this parameter remained unchanged in CD (**Table 2**). Conversely, FFM% increased in MD and remained unchanged in CD (**Table 2**).

3.3. Glycemic profile and calcium homeostasis

MD final serum 25-hydroxyvitamin D levels and HOMA2-B% were higher and final serum uric acid, HbA1c, and PTH levels were lower compared with baseline values (**Table 2**). A comparison of changes over 12 weeks to the baseline (deltas) revealed that serum uric acid, fasting glucose, HbA1c, PTH, and Mg decreased, whereas HOMA2-B% and 25-hydroxyvitamin D increased in MD compared to CD (**Table 3**).

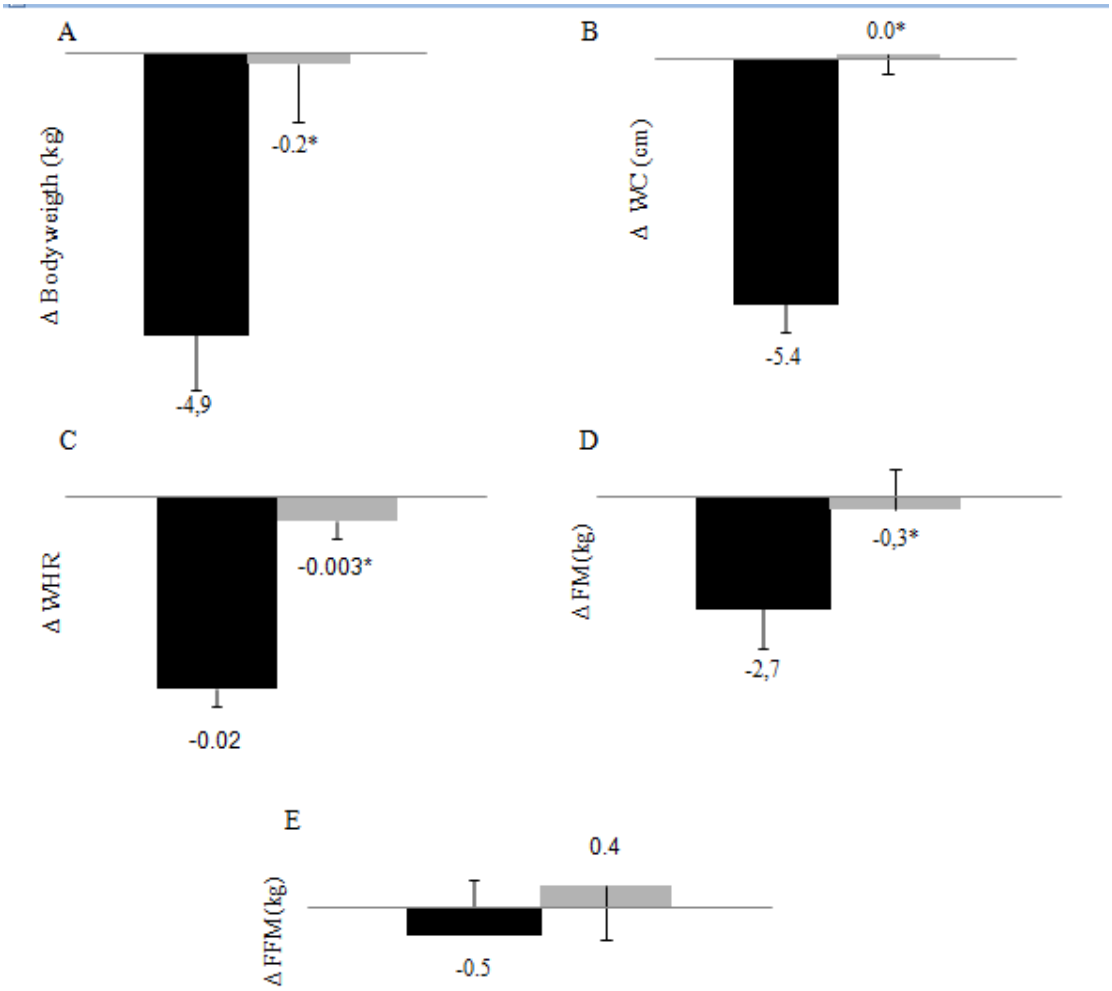

Figure 2 – Mean (SEM) body weight (A), waist circumference (WC) (B), waist-hip ratio (WHR) (C), fat mass (FM) (D), and fat-free mass (FFM) (E) changes from baseline according to study phase (N = 14). During consecutive 12 weeks subjects consumed a fat-free milk (700 mg of Ca/d) or a low-Ca control (6.4 mg of Ca/d) shake for breakfast. Energy restricted diets (-500kcal/d, 800 mg of Ca/d) were prescribed. Except for FFM, all variables differed between phases (*P <0.025, paired t-test with Bonferroni correction for multiple comparisons). Delta (Δ) was calculated subtracting the final value (after 12 week-intervention) from the baseline value.

Table 2 – Food intake, anthropometry and body composition, and biochemical data at baseline and during high-calcium fat-free milk (MD) and low-calcium control (CD) experimental phases (N=14)[1]

Food intake[2]	**Baseline**	**MD**	**CD**
Energy (kcal/d)	1637.3 ± 480.6	1682.9 ± 417.0	1715.5 ± 438.9
Carbohydrate (g/d)	216.1 ± 69	233.5 ± 52.8	237.5 ± 57.5
Fiber (g/d)	18.1 ± 5.5	24.6 ± 4.8**	15.2 ± 6.7
Protein (g/d)	67.3 ± 21.2	80.4 ± 21.2	79.4 ± 23.3
Total fat (g/d)	54.8 ± 23.4	44.1 ± 15.2	45.2 ± 15.8
MUFA(g/d)	15.2 ± 8.1	12.4 ± 6.0	12.8 ± 5.5
PUFA(g/d)	10.4 ± 4.5	8.2 ± 3.0	7.9 ± 3.2
SFA (g/d)	13.5 ± 7.2	9.5 ± 4.0*	10.4 ± 4.1
Calcium (mg/d)	488.9 ± 233.4	1218.3 ± 145.1**	524.8 ± 300.6
Magnesium (mg/d)	175.6 ± 56.8	239.8 ± 55.2*	197.3 ± 103.9
Phosphorus (mg/d)	838.2 ± 252.7	1642.7 ± 265.4*	841.5 ± 287.5
Zinc (mg/d)	6.5 ± 2.9	5.8 ± 2.9	6.0 ± 2.3
Calcium/Phosphorus ratio	0.57 ± 0.1	0.74 ± 0.0	0.62± 0.1
Anthropometry and body composition			
Body weight (kg)	77.3 ± 14.9	73.5 ± 15.8*	75.9 ± 15.5
BMI (kg/m^2)	29.4 ± 4.5	27.9 ± 4.4	28.9 ± 4.8
FM (%)	34.1 ± 6.0	33.1 ± 6.7*	33.6 ± 6.6
FFM (%)	65.9 ± 6.0	66.9 ± 6.7*	66.4 ± 6.6
WC (cm)	95.1 ± 11.4	91.9 ± 11.7*	93.1 ± 12.2
WHR	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
Biochemical data			
Uric acid (mg/dL)	3.3 ± 0.7	3.2 ± 0.8*	3.5 ± 1.1
Fructosamine (µmol/L)	172.1 ± 101.6	153.4 ± 84.5	183.4 ± 104.3
Fasting glucose (mg/dL)	127.4 ± 42.0	123.4 ± 39.6	150.3 ± 59.2
HbA1c (%)	7.1 ± 1.4	6.4 ± 1.1*	6.9 ± 1.5
Insulin (µUI/mL)	8.1 ± 3.6	9.2 ± 5.1	8.6 ± 4.1
HOMA2 %B	57.2 ± 31.5	61.9 ± 35*	45.7 ± 27.9
HOMA2 %S	112.1 ± 55.1	108.8 ± 71.1	104.2 ± 54.3
HOMA2-IR	1.1± 0.5	1.3 ± 0.7	1.2 ± 0.6
Calcium (mg/dL)	9.0 ± 0.4	9.4 ± 0.5	8.9 ± 0.5
PTH (pq/mL)	37.9 ± 15.6	30.6 ± 10.1*	37.4 ± 13.2
25-OH vitamin D (ng/mL)	27.3 ± 7.1	35.6 ± 11.7*	27.7 ± 8.4
Phosphorus (mg/dL)	3.5 ± 0.3	3.6 ± 0.3	3.3 ± 0.2
Magnesium (mg/dL)	1.8 ± 0.2	1.7 ± 0.3	1.9 ± 0.2

[1] Values are mean ± SD.[2] Food intake was assessed by 3-d food records. Different from baseline: ** $P \leq 0.05$; ** $P \leq 0.01$. Calculated from t-paired test for the comparisons between MD and CD phases, with baseline value included as a covariate (N = 14). MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; BMI, body mass index; FM, fat mass; FFM, free-fat mass; WC, waist circumference; WHR, waist-hip ratio; HbA1c, glycated hemoglobin; HOMA2-IR, Homeostasis Model Assessment-2 of Insulin Resistance; HOMA2% B, HOMA beta-cell function (% B); HOMA2% S, HOMA insulin sensitivity (% S); PTH, parathyroid hormone;25-OH vitamin D, 25-hydroxyvitamin D.

Table 3 – Food intake and biochemical data changes from baseline[†] in response to high-calcium fat-free milk (MD) and low-calcium control experimental phase (CD) (N=14)[1]

	Study phase			
Food intake[‡]	**MD**		**CD**	
	Mean	**SD**	**Mean**	**SD**
Energy (kcal/d)	-62.1	501.6	186.4	214.4
Carbohydrate (g/d)	9.9	65.3	28.9	41.4
Fiber (g/d)	7.3	5.1	-3.9	4.6*
Protein (g/d)	19.9	26.1	13.4	14.3
Total fat (g/d)	-20.7	32.7	-20.4	13
MUFA (g/d)	-6.1	12.0	0.9	3.5
PUFA (g/d)	-4.4	6.8	-0.5	3.1
SFA (g/d)	-7.3	10.0	0.2	4.0
Calcium (mg/d)	830.0	178.2	-64.8	368.5*
Phosphorus (mg/d)	854.5	217.0	-47	303.7*
Magnesium (mg/d)	65.3	53.2	20.3	105.8
Zinc (mg/d)	-0.7	1.9	-0.5	2.1
Biochemical data				
Uric acid (mg/dL)	-0.4	0.5	0.5	0.8*
Fructosamine(µmol/L)	-15.1	27.9	15.3	52.4
Fasting glucose (mg/dL)	-7.7	17.6	29.3	44.5*
Hb1Ac (%)	-0.7	1.0	0.6	0.7*
Insulin (µUl/mL)	0.7	4	0.9	3.4
HOMA2-IR	0.06	0.5	0.2	0.5
HOMA2 %B	7.3	23.9	-13.8	34.1*
HOMA2 %S	10.9	55	-13.1	33.1
Calcium (mg/dL)	0.3	0.7	-0.03	0.4
PTH (pq/mL)	-12.8	13.1	5.6	9.9*
25-OH vitamin D (ng/mL)	11.0	10.2	-2.4	3*
Phosphorus (mg/dL)	0.14	0.33	-0.18	0.43
Magnesium (mg/dL)	-0.3	0.3	0.1	0.2*

MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; HbA1c, glycated hemoglobin; HOMA2-IR, Homeostasis Model Assessment-2 of Insulin Resistance; HOMA2% B, HOMA beta-cell function (% B); HOMA2% S, HOMA insulin sensitivity (% S); PTH, parathyroid hormone; 25-OH vitamin D, 25-hydroxyvitamin D.

[†]Changes from baseline were calculated by substracting final value from the baseline value. [‡]Food intake was assessed by 3-d food records. Complete nutritional information was available only for the nutrients listed on DietPro® software or on food labels. Some nutrient contents not listed, including vitamin D, were not available. *P<0.025. P-value was estimated by paired t-test or Wilcoxon rank sum (both with Bonferroni correction for multiple comparisons).

3. Discussion

In the present study, the consumption of ~3 servings of fat-free milk and 1,200 mg of dietary Ca/d (the amount consumed in MD phase) was more effective to control obesity (body weight, FM, and WC reduction), and the parameters related with the glycemic control (decrease in serum uric acid, HbA1c, and fasting glucose and increase in HOMA2-B%) than the control diet (low-Ca consumption, ~525 mg/d) in individuals with T2DM and low habitual Ca intake (less than 600 mg/d). Possible mechanisms that explain our results are summarized in **Figure 3.** Consumption of fat-free dairy (MD) led to a decrease of about 6% in body weight (approximately 5 kg), while the low-Ca (CD) did not change body weight. This result suggests that increased fat-free dairy intake can be a good strategy to control body weight, reducing FM, and improving the glycemic control.

Some authors reported that diets higher in dairy lead to beneficial effects on weight loss (Zemel et al., 2004), body fat (Zemel et al., 2005; Zemel et al., 2009; Torres and Sanjuliani, 2013) and WC reduction (Zemel et al., 2005; Zemel et al., 2009; Stancliffe et al., 2011; Torres and Sanjuliani, 2013), and insulin sensitivity increase (Rideout et al., 2013; Maki et al., 2015), while others have observed no effects in these parameters (Bowen et al., 2005; Gunther et al., 2005; Harvey-Berino et al., 2005; Thompson et al.,2005; Jones et al., 2013). Methodological differences in these clinical trials can explain these controversial results, as described below.

First, the Ca dose (1.100 (Zemel et al., 20014) to 2.400 mg of Ca/d (Bowen et al., 2005)) and the supplementation period (six weeks (Maki et al., 2015) to one year (Gunther et al., 2005; Rideout et al., 2013)) differed greatly between studies. However, the habitual Ca intake of the participants seems to have been more decisive in the results than the doses or supplementation periods. Some authors did not report the habitual Ca intake of the participants (Gunther et al., 2005; Thompson et al., 2005). In other cases, daily habitual Ca consumption was greater than or between 700 and 800 mg/d (Bowen et al., 2005; Harvey-Berino et al., 2005; Jones et al., 2013). In these studies, no effect on adiposity or glycemic profile was verified (Bowen et al., 2005; Gunther et al., 2005; Harvey-Berino et al., 2005; Thompson et al.,2005; Jones et al., 2013). On

the other hand, when habitual Ca intake at baseline was less than or between 600 and 700 mg/d, beneficial effects on body composition and on insulin sensitivity were observed (Zemel et al., 2004; Zemel et al., 2005; Zemel et al., 2009; Stancliffe et al., 2011; Torres and Sanjuliani, 2013; Rideout et al., 2013; Maki et al., 2015). Zemel et al. (2009) emphasized the importance of selecting individuals with low habitual intake of Ca (less than 600 mg/d) for the benefits of increased Ca consumption to occur. Our participants consumed 488.9 (SD of 233.4) mg of Ca/d at baseline (Table 2).

Furthermore, studies comparing the effects of a high intake of dairy versus a control diet (low in dairy products) have showed significant reduction on body weight and on central fat when participants were concomitantly submitted to energy restriction (Zemel et al., 2004; Zemel et al., 2005; Zemel et al., 2009; Torres and Sanjuliani, 2013). The beneficial effects of increased Ca intake seem to be more significant when it is associated with moderate energy intake reduction (Abargouei et al., 2012). However, in our study we observed that although the energy restriction prescribed was not followed by the subjects, the increased consumption of fat-free milk enhanced weight loss, improved body composition, and promoted glycemic control. Aside from this, consumption of low-fat dairy seems to have more effect on T2DM control than the consumption of whole dairy (Candido et al., 2013). We and other authors have also observed positive effects of Ca supplementation through low-fat dairy on body composition and on glycemic profile (Zemel et al., 2005; Zemel et al., 2009; Torres and Sanjuliani, 2013; Rideout et al., 2013; Maki et al., 2015).

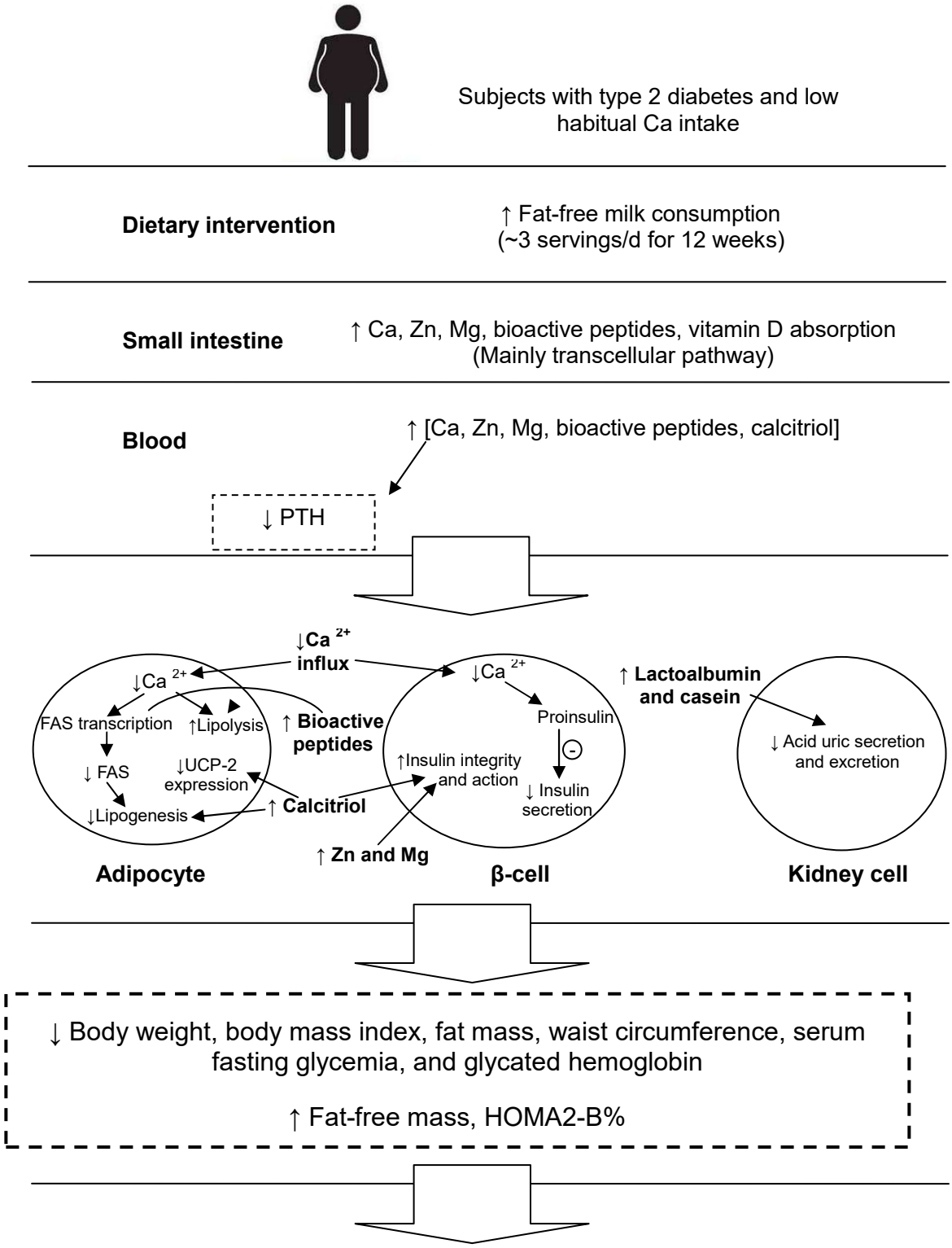

Figure 3 – Possible mechanisms that explain the effects of increased fat-free milk consumption on diabetes and obesity control, based on our results. Ca, calcium; FAS, fatty acid synthase; HOMA2-B%, Homeostasis Model

Assessment - beta-cell function; Mg, magnesium; PTH, parathormone; UCP-2, uncoupling protein 2; Zn, zinc.

In most of the studies in which dairy Ca did not affect body weight, adiposity, and IR, habitual Ca intake was equal or higher than 700 mg/d (Bowen et al., 2005; Gunther et al., 2005; Harvey-Berino et al., 2005; Thompson et al.,2005; Jones et al., 2013). Although some authors tested the effect of energy restriction (Bowen et al., 2005; Harvey-Berino et al., 2005; Thompson et al., 2005; Jones et al., 2013) and/or of low-fat dairy consumption (Bowen et al., 2005; Harvey-Berino et al., 2005; Thompson et al., 2005), the habitual Ca consumption seems to be the main determinant of the outcomes. Other features, such as the use of low vitamin D content dairy products (Bowen et al., 2005), lack of statistical power (Harvey-Berino et al., 2005), and intervention based only on nutritional counselling and using food records to assess food intake (since dairy was not provided in the laboratory, there is no guaranty that the treatment was actually applied) (Gunther et al., 2005) may also partially explain the lack of positive effects in some studies.

About 25-35% of ingested Ca is absorbed in the intestine via paracellular (passive transport) and transcellular (active transport) pathways (Kopic and Geibel, 2013). Transcellular Ca absorption is mediated by calcitriol and occurs mainly in duodenum and jejunum (Kopic and Geibel, 2013). The rate of paracellular pathway is almost constant, while the transcellular pathway is more efficient under conditions of dietary Ca restriction (Kopic and Geibel, 2013). Our participants were low habitual Ca consumers (<600 mg/d), so probably the transcellular Ca uptake increased when they consumed more Ca (MD phase). So, adding 3 servings of fat-free milk per day (MD phase) could be of interest in clinical practice, since Western diet is typically rich in sodium and P (minerals that reduce Ca absorption), and poor in Ca and vitamin D (Calvo and Tucker, 2013). However, the long-term effect is unknown, since the paracellular Ca absorption tends to increase under normal Ca consumption (Kopic and Geibel, 2013).

Possible mechanisms involving Ca on weight loss and glycemic control are not clear. It has been suggested that a low-Ca intake increases serum PTH

and calcitriol (1,25-dihydroxyvitamin D), resulting in an Ca^{2+} influx into adipocytes via either receptor- or voltage-mediated Ca^{2+} channel activation (Zemel et al., 2000). High intracellular $[Ca^{2+}]i$ stimulates fatty acid synthase (FAS) gene expression, and consequently results in stimulation of FAS activity, increasing *de novo* lipogenesis (Zemel et al., 2000). In addition, elevated $[Ca^{2+}]i$ activates Ca-dependent kinases and Ca/calmodulin-dependent protein kinase, which phosphorylate phosphodiesterase, resulting in reduced cAMP levels and, consequently, in inhibition of hormone-sensitive lipase (Larsson et al., 2016). Consequently, a low-Ca diet induces lipogenesis and inhibits lipolysis, leading to body fat accumulation and IR (Zemel et al., 2000). On the other hand, a high-Ca intake seems to reduce lipogenesis and promote lipolysis and increased thermogenesis, increasing fat oxidation and energy expenditure (Zemel et al., 2000). In our study, the breakfast shakes offered in both phases contained 3.5 mcg of vitamin D, which is about 20% of the recommendation for adults (IOM, 2011). However, it seems that vitamin D from dairy shake (MD) was more bioavailable than vitamin D3 supplementation (CD), since serum vitamin D only increased in MD. The role of calcitriol in lipogenesis has been discussed. Concomitant supplementation of Ca and vitamin D increases intestinal absorption of Ca (Candido and Bressan, 2014) and body weight loss (Pittas et al., 2007). In adipocytes, vitamin D suppresses the differentiation of preadipocytes, reducing lipogenesis (Candido and Bressan, 2014). We observed a reduction on serum PTH and an increase in serum 25-hydroyvitamin D after MD, suggesting the occurrence of these mechanisms. Other beneficial effects of dietary Ca, such as appetite regulation (Lorenzen et al., 2007), reduction of central obesity mediated by cortisol (Zemel et al., 2004; Zemel et al., 2005), and changes in intestinal microbiota (Gomes et al.,2015) have been suggested, but have not been confirmed yet.

Our breakfast shakes contained 17.1 (SD 0.2) g of protein. However, MD shakes contained 20% casein and 80% whey protein, while CD shakes contained 100% whey protein. We included only whey protein in the CD because micellar casein (as present in milk) could not be used since it contains Ca (Gaucheron, 2005) and hydrolyzed casein is less bioavailable (Dugan and Fernandez, 2014). In milk, about 30% of Ca exists as free ionic Ca and the

remaining ~70% is complexed with casein in micellar Ca phosphate (Gaucheron, 2005). Besides, hydrolysed casein coagulates in the stomach, being less available for enzymatic hydrolysis and less absorbed in the intestine (Dugan and Fernandez, 2014). Milk proteins and bioactive peptides have been associated with increased satiety, increased thermogenesis, muscle protein loss sparing, and enhanced glycemic control (Acheson et al., 2011). Branched chain amino acids (BCAAs), especially leucine, seem to increase body fat loss by increasing fat oxidation, stimulating muscle protein synthesis, and thus reducing lean tissue loss (Sun and Zemel, 2007). Although casein and whey protein have similar amounts of leucine, when whey protein is ingested alone, its rapid intestinal transit reduces BCAAs absorption (Eller et al., 2010). When whey protein is ingested with casein, which occurs naturally in milk protein, it increases intestinal transit time favouring BCAAs absorption (Eller et al., 2010). Therefore, in our study, the casein present in milk may have been essential to increase the uptake of leucine, which may have contributed to the preservation of lean tissue and the reduction of body fat in MD.

The effects of Ca on glycemic control seem to be associated with insulin secretion, since Ca stimulates the conversion of proinsulin into insulin and promotes insulin release by the pancreatic beta cells (Pittas et al., 2007). Thus, an inadequate Ca intake may alter the balance between extracellular and intracellular beta cell Ca pools, which may interfere with the secretory function of pancreatic beta cells (Pittas et al., 2007). In the present study, the increase in HOMA2-B% suggests an increase in insulin secretion after MD phase. HOMA2-B% has been considered the main determinant of glycemic control in T2DM adults (Monnier et al., 2006). Additionally, MD phase decreased HbA1c concentrations compared to baseline and to CD phase, improving the glycemic control of our participants. This reduction can be due to the direct (i.e. stimulation of insulin secretion) and indirect (i.e. body weight loss and reduction of FM) effects of Ca on insulin sensitivity. Other milk components, such as Zn, Mg, and vitamin D play a key role on insulin action. Zn is involved in the synthesis, storage, and secretion of insulin as well as the conformational integrity of insulin in a hexametric form (Yahya et al., 2011). Mg acts on insulin secretion and is a cofactor for multiple enzymes involved in carbohydrate

metabolism (Yahya et al., 2011). In pancreatic beta cells, vitamin D activates transcription of the insulin gene and the insulin receptor gene (Candido and Bressan, 2014). In addition, the presence of a vitamin D response element in the human insulin gene promoter suggests a potential influence of vitamin D on glucose homeostasis (Candido and Bressan, 2014). Therefore, our results cannot be attributed to the Ca from dairy only, as the synergistic effects of the different components of fat-free dairy may have influenced our results. The amount of Ca added to MD shakes (700 mg) was established bearing in mind the need to offer a relatively high load of Ca without affecting its applicability in clinical practice. These shakes were very well tolerated by the subjects. However, we cannot assure that the results obtained in this study will be observed if instead of being consumed once that same amount of Ca was consumed in small amounts throughout the day.

We demonstrated that MD phase reduced serum acid uric concentrations compared to baseline and to CD phase. Milk proteins (lactalbumin and casein) have uricosuric effect (Ghadirian et al., 1995). In diabetic patients, serum acid uric has been considered an early marker of impaired glucose metabolism and a good predictor of cardiovascular risk (Verdoia et al., 2014). Hyperglycemia and hyperinsulinemia resulting from IR declines renal function, and increases uric acid production (Neupane et al., 2016). Concomitantly, high serum uric acid concentrations inhibit insulin signaling and induce IR (Zhu et al., 2014). Uric acid also inhibits endothelial nitric oxide bioavailability and activates rennin-angiotensin system, resulting in renal dysfunction, hypertension and dyslipidemia (Neupane et al., 2016). In our study, the MD phase decreased HbA1c and increased HOMA2-B%, suggesting that the attenuation of IR contributed to lower serum acid uric concentrations.

Dietary fiber intake increased after MD phase. However, the recommendation for dietary fiber (14 g fiber/1,000 kcal) (ADA, 2015) was achieved only on the MD phase. The difference in dietary fiber between the experimental diets was approximately 9.4 g/d after the interventions, since MD consumption was equivalent to 24.6 (SD 4.8) g/d whereas CD was 15.2 (SD 6.7) g/d. An increase in fiber intake equivalent to 10g/d contributes to a weight loss of only 39 g/year (Du et al., 2010) and foods rich in fiber can affect

glycemic control when the intake is equivalent to 42.5 g of fiber/d (Silva et al., 2013), which refutes the possibility that fiber interfered in our results.

In our study, the dietary Ca:P ratio was lower than the recommended (≥ 1:1) (Calvo and Park, 1996), both at baseline and after our experimental phases. This ratio increased in MD, as the baseline ratio was 0.49 and the final was 0.74, unlike what happened on CD, where the baseline ratio was 0.66 and the final was 0.62. In general, Western diets are high in P and low in Ca (Calvo and Tucker, 2013). A high P associated with a lower Ca consumption may have adverse effects on the skeleton, since it raises serum PTH and thereby reduces bone mineral density (Adatowvor et al., 2015). However, an adequate Ca intake seems to counteract this effect of excess P (Lee and Cho, 2015), as observed in our study. Kemi et al. (2010) have suggested that only diets with the Ca:P ratios below 0.50 are able to negatively affect Ca metabolism and bone mineral density.

5. Conclusion

We conclude that about 3 servings of fat-free milk (700 mg of dietary additional Ca/d) and 1,200 mg of dietary Ca/d for 12 weeks promoted greater weight loss and glycemic control in individuals with T2DM and low-habitual Ca consumption (<600 mg/d) than did the low-Ca diet (~525 mg/d). These findings may be useful in the dietary treatment of T2DM.

6. Acknowledgments

We thank Instituto Federal do Sudeste de Minas (IF Sudeste MG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Bioclin, Quibasa Basic Chemical Ltda. None of the authors had any personal or financial conflict of interest related to this study. The authors' contributions were as follows: J.M.G.G. designed the study, analyzed and interpreted the data, and wrote the manuscript. J.A.C. assisted in analyzing the data. R.C.G.A.designed the study, interpreted the data, and edited the manuscript. All authors read and approved the final manuscript.

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5.2. ARTICLE 2

Increased Fat-Free Milk Consumption, Metabolic Syndrome, and Cardiometabolic Outcomes in Adults with Type 2 Diabetes Mellitus

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Abstract: 250 words

Main text: 3375 words

References: 33

Figures: 2

Tables: 2

Short-title: Dairy, metabolic syndrome and cardiovascular disease

Keywords: Calcium; dairy; diet; metabolic syndrome; cardiovascular disease; lipids; blood pressure

Abstract

Background and Aim

Dairy consumption, the main source of dietary calcium (Ca), is inversely associated with obesity, inflammation, hyperlipidaemia and type 2 diabetes mellitus (T2DM). However, the effect of the consumption of increased Ca from fat-free milk on metabolic syndrome (MetS) and cardiometabolic outcomes in adults with T2DM has not been assessed yet. Therefore, we investigated the effects of high-Ca fat-free milk phase (MD) (prescription of ~1,500 mg of Ca/d) *vs.* low-Ca phase (CD) (prescription of ~800 mg of Ca/d) on MetS and cardiometabolic measures in individuals with T2DM and low habitual Ca consumption (<600 mg/d).

Methods and Results

In this randomized crossover design, 14 adults with T2DM (49.5 $\pm$ 8.6 years, body mass index 29.4 $\pm$ 4.5 kg/m^2) consumed either MD or CD for 12 weeks, with a washout of eight weeks between phases. A breakfast shake containing 700 mg (MD) or 6.4 mg (CD) of Ca was consumed in the laboratory. Additionally, energy restricted diets were prescribed (800 mg of dietary Ca/d). Waist circumference (WC), fasting glucose, fasting triglycerides (TG), systolic (SBP) and diastolic blood pressure (DBP), fasting total cholesterol, fasting low-density lipoprotein cholesterol (LDL-c), fasting high-density lipoprotein cholesterol (HDL-c), HDL/LDL ratio, HDL/TG ratio and lipid accumulation product (LAP) index were assessed at baseline and after each phase. Ca consumption during the study was equivalent to 1,200 mg/d during MD and of 525 mg/d during CD. There was a greater reduction in WC, SBP and DBP, and LAP index after MD compared with CD. HDL/LDL ratio increased and total cholesterol, LDL-c, SBP and DBP, and LAP index decreased only in MD.

Conclusion

The consumption of ~1,200 mg of Ca/d (700 mg from fat-free milk + 500 from other dietary sources) decreased some MetS components and cardiometabolic measures adults with T2DM.

Registration: ClinicalTrials.gov NCT02377076

1. Introduction

Dairy products are source of nutrients and bioactive compounds, which may reduce inflammation and oxidative stress, besides favoring glycemic control, and body weight loss (Visioli and Strata, 2014). Low-fat dairy is an excellent source of protein, calcium (Ca), zinc (Zn), phosphorus (P), magnesium (Mg), and vitamin D (Dugan and Fernandez, 2014). Milk proteins, particularly whey protein, seem to have insulinotrophic, hypolipidemic, antihypertensive, and antiinflammatory properties (Dugan and Fernandez, 2014). Increased Ca consumption has been associated with beneficial effects on body weight (Jacobsen et al., 2005), blood pressure (BP) (Wang et al., 2008), and type 2 diabetes mellitus (T2DM) (Tong et al.,m 2011). Other micronutrients of dairy, such as vitamin D, Zn, P, and Mg are associated with lower T2DM and cardiovascular diseases risk (Rice et al., 2013).

Large studies, such as the Multi-Ethnic Study of Atherosclerosis, have shown inverse associations between low-fat dairy consumption and inflammation and oxidative stress biomarkers concentrations, which play major roles in the onset and development of MetS and its components (Nettleton et al., 2006). Increased dairy consumption has also been associated with lower incidence of metabolic syndrome (MetS), and T2DM (Visioli and Strata, 2014). Dairy products seem to modulate some cardiovascular risk factors (e.g., insulin response, dyslipidemia, oxidative stress and inflammation markers, BP, etc.), but the association between dairy consumption and cardiovascular disease is controversial (Visioli and Strata, 2014; Alexander et al., 2016). Apparently, the consumption of energy restricted diets containing low-fat dairy products seem be more efficient in reducing body weight, body fat mass, waist circumference (WC), and BP compared with whole dairy and non-energy restricted diets (Abargouei et al., 2012; Ralston et al., 2012).

In a review study involving data from randomized clinical trials (RCTs), Pasin and Comerford (2015) concluded that dairy foods might improve glycemic status in adults with T2DM. However, these results were observed in acute studies or in long-term studies that used probiotic yogurt for the intervention group (Pasin and Comerford, 2015). The microorganisms present in yogurt can improve systemic disorders, such as T2DM, dyslipidemia, inflammation, and

oxidative stress (Pasin and Comerford, 2015). So, the effects of other dairy products (i.e., milk) on metabolic profile of T2DM adults need to be better explored.

Whilst previous studies have investigated the effect of dairy products on body weight and glycemic control (Zemel et al., 2005; Torres et al., 2013; Visioli and Strata, 2014; Pasin and Comerford, 2015), to the best of our knowledge no other randomized clinical trial has assessed the effect of increased Ca consumption from fat-free milk associated with an energy restricted diet on MetS and cardiometabolic outcomes in adults with T2DM. Considering the relevance of dietary treatment on T2DM and MetS, it is necessary to verify if diets containing high-Ca from fat-free milk have beneficial effects on these disorders. Therefore, this study aimed to investigate the effect of increased Ca consumption from dairy on MetS components and cardiometabolic measures in adults with T2DM.

2. Participants and Methods

Described on pages 70-84, “PARTICIPANTS AND METHODS”.

3. Results

3.1. Participants

Sixteen subjects met the eligibility criteria. However, 14 (4 men and 10 women, BMI of 29.4 $\pm$ 4.5 kg/m^2, and 49.5 $\pm$ 8.6 years of age) (**Table 2)** completed both phases and were included in the analyses (**Figure 2**).

3.2. Dietary intake

Dietary fiber, Ca, Mg, and P intake increased in MD compared with CD (**Table 2**). Ca and P consumption at the end of MD were 232% (693 mg) and 195.3% (801 mg) higher, respectively, compared with the end of the CD phase (**Table 2**). The Ca:P ratio was 0.49 and 0.66 at baseline, and 0.74 and 0.62 post-treatment for MD and CD, respectively. No significant changes in dietary energy, carbohydrate, protein, fat, Mg, and Zn intake were observed between the two dietary phases (**Table 2**).

3.3. MetS Components and Cardiometabolic Measures

WC decreased after MD while no change was observed after CD (**Table 2**). Reduction in WC from baseline to 12-week were greater in MD than CD (**Table 2**). Total cholesterol, LDL-c, SBP and DBP, and LAP index decreased after MD (**Table 2**). HDL/LDL ratio was higher after MD (**Table 2**). There were no changes on biochemical variables in the CD, except from an increase in fasting glucose (**Table 2**). Comparing changes from baseline to 12-week intervention, SBP, DBP, and LAP index decreased in MD compared with CD (**Table 2**).

3.4. Calcium Homeostasis Markers

Serum Ca and P remained unchanged in both phases (**Table 2**). Serum Mg and PTH decreased after MD, but there was no change after CD (**Table 2**). Serum 25-hydroxyvitamin D was higher after MD and lower after CD (**Table 2**). Changes in vitamin D, Mg and PTH from baseline to 12-week intervention differed between dietary phases (**Table 2**).

Table 2 – Food intake, MetS components and other cardiometabolic outcomes presented by the subjects, according to study experimental phase (N=14) [a]

	MD phase			CD phase		
	Baseline	**Final**	**Change from baseline**	**Baseline**	**Final**	**Change from baseline**
Nutrient [b]						
Energy (kcal/d)	1745. ± 504.6	1682.9±417.0	-62.1 ± 501.6	1529.1 ± 456.7	1715.5 ± 438.9	186.4 ± 214.4
Carbohydrate (g/d)	223.5 ± 72.7	233.5 ± 52.8	9.9 ± 65.3	208.6 ± 65.3	237.5 ± 57.5	28.9 ± 41.4
Fiber (g/d)	17.3 ± 4.3	24.6 ± 4.8*	7.3 ± 5.1	19.0 ± 6.7	15.2 ± 6.7*	-3.9 ± 4.6†
Protein (g/d)	68.7 ± 20.8	80.4 ± 21.2	11.7 ± 26.1	66.0 ± 21.6	79.4 ± 23.3	13.4 ± 14.3
Total fat (g/d)	64.8 ± 29.8	44.1 ± 15.2	-20.7 ± 32.7	44.9 ± 17.1	45.2 ± 15.8	-0.3 ± 13
MUFA(g/d)	18.5 ± 10.1	12.4 ± 6.0	-6.1 ± 12.0	12.0 ± 6.1	12.8 ± 5.5	0.9 ± 3.5
PUFA(g/d)	12.6 ± 6.0	8.2 ± 3.0	-4.4 ± 6.8	8.3 ± 3.1	7.9 ± 3.2	-0.5 ± 3.1
SFA (g/d)	16.8 ± 9.9	9.5 ± 4.0*	-7.3 ± 10.0	10.2 ± 4.6	10.4 ± 4.1	0.2 ± 4.0
Calcium (mg/d)	388.4 ± 184.2	1218.3 ± 145.1	830.0 ± 178.2	589.5 ± 282.7	524.8 ± 300.6	-64.8± 368.5†
Phosphorus (mg/d)	788.2 ± 217.0	1642.7±265.4*	854.5±217.0	888.2 ± 288.4	841.5 ± 287.5	-46. ± 303.7†
Magnesium (mg/d)	174.4 ± 47.4	239.8 ± 55.2*	65.4 ± 55.2	176.9 ± 66.2	197.3 ± 103.9	20.4 ± 105.8
Zinc (mg/d)	7.2 ± 3.0	8.5 ± 3.3	-0.7 ± 1.9	5.8 ± 2.9	5.7 ± 2.3	-0.5 ± 2.1
MetS components						
WC (cm)	97.0 ± 11.1	91.9 ± 11.7*	-5.1 ± 2.4	93.1 ± 11.7	93.1 ± 12.2	0.0 ±1.5†
Fasting glucose (mg/dL)	131.1 ± 42.9	123.4 ± 39.6	-7.7 ± 17.6	123.8 ± 41.1	150.3 ± 59.2*	26.5 ± 44.5

	MD phase			CD phase		
	Baseline	**Final**	**Change from baseline**	**Baseline**	**Final**	**Change from baseline**
Fasting TG (mg/dL)	168.6 ± 98.5	153.4 ± 84.5	-15.2 ± 66.2	175.6 ± 104.7	183.4 ± 104.3	7.7 ± 62.5
HDL-c (mg/dL)	43.0 ± 8.1	43.5 ± 9.0	0.5 ± 4.1	45.0 ± 10.8	47.4 ± 12.1	2.4 ± 5.5
SBP (mmHg)	136.6 ± 12.7	120.6 ± 15.8*	-16.0 ± 11.9	124.1 ± 10.6	123.3 ± 12.2	-0.8 ± 10.9†
DBP (mmHg)	84.2 ± 7.9	73.4 ± 9.9*	-10.8 ± 8.3	77.1 ± 8.9	75.8 ± 7.9	-1.3 ± 7.6†
Other cardiometabolic outcomes						
Total cholesterol (mg/dL)	181.2 ± 24.6	161.7 ± 18.3*	-19.5 ± 24.4	178.7 ± 31.8	180.9 ± 21.5	2.1 ± 33.5
LDL-c (mg/dL)	104.5 ± 22.5	87.5 ± 17.2*	-17.0 ± 17.2	96.4 ± 28.7	96.8 ± 26.0	0.3 ± 26.5
VLDL-c (mg/dL)	33.7 ± 19.7	30.7 ± 16.9	-3.0 ± 13.2	35.1 ± 20.9	36.7 ± 20.9	1.5 ± 12.5
HDL/LDL ratio	0.4 ± 0.1	0.5 ± 0.2*	0.1 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.0 ± 0.2
HDL/TG ratio	0.4 ± 0.3	0.4 ± 0.3	0.0 ± 0.1	0.4 ± 0.3	0.4 ± 0.5	0.1 ± 0.2
LAP index (cm.mmol/L)	71.9 ± 42.9	23.6 ± 13.1*	-48.4 ± 51.1	22.2 ± 13.6	20.9 ± 11.5	-1.2 ± 7.4†
Calcium homeostasis markers						
Calcium (mg/dL)	9.1 ± 0.4	9.4 ± 0.5	0.3 ± 0.7	8.9 ± 0.3	8.9 ± 0.5	-0.0 ± 0.4
PTH (pq/mL)	43.4 ± 18.6	30.6 ± 10.1*	-12.8 ± 13.1	32.4 ± 12.7	37.4 ± 13.2	5.6 ± 9.9†
25-OH vitamin D (ng/mL)	24.6 ± 6.5	35.6 ± 11.7*	11.0 ± 10.2	30.1 ± 7.6	27.7 ± 8.4*	-2.4 ± 3.0†
Phosphorus (mg/dL)	3.5 ± 0.3	3.6 ± 0.3	0.1 ± 0.3	3.5 ± 0.4	3.3 ± 0.2	-0.2 ± 0.4
Magnesium (mg/dL)	1.8 ± 0.3	1.5 ± 0.2*	-0.3 ± 0.3	1.8 ± 0.2	1.9 ± 0.2	0.1 ± 0.2†

[a] Values are means± SD. [b] Food intake was assessed by 3-d food records. MetS, metabolic syndrome; MD, high-calcium fat-free milk phase; CD, low-calcium control (CD) phase; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; WC, waist circumference; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL-c, low-density lipoprotein cholesterol; VLDL-c, very low-density lipoprotein cholesterol; LAP, lipid accumulation product; PTH, parathyroid hormone;25-OH vitamin D, 25-hydroxyvitamin D.

*P<0.05. P-value was estimated by t-paired test, with baseline value included as covariate (N = 14). (intragroup comparisons). [†]P<0.025. P-value was estimated by paired t-test or Wilcoxon rank sum (both with Bonferroni correction for multiple comparisons) (intergroup comparisons).

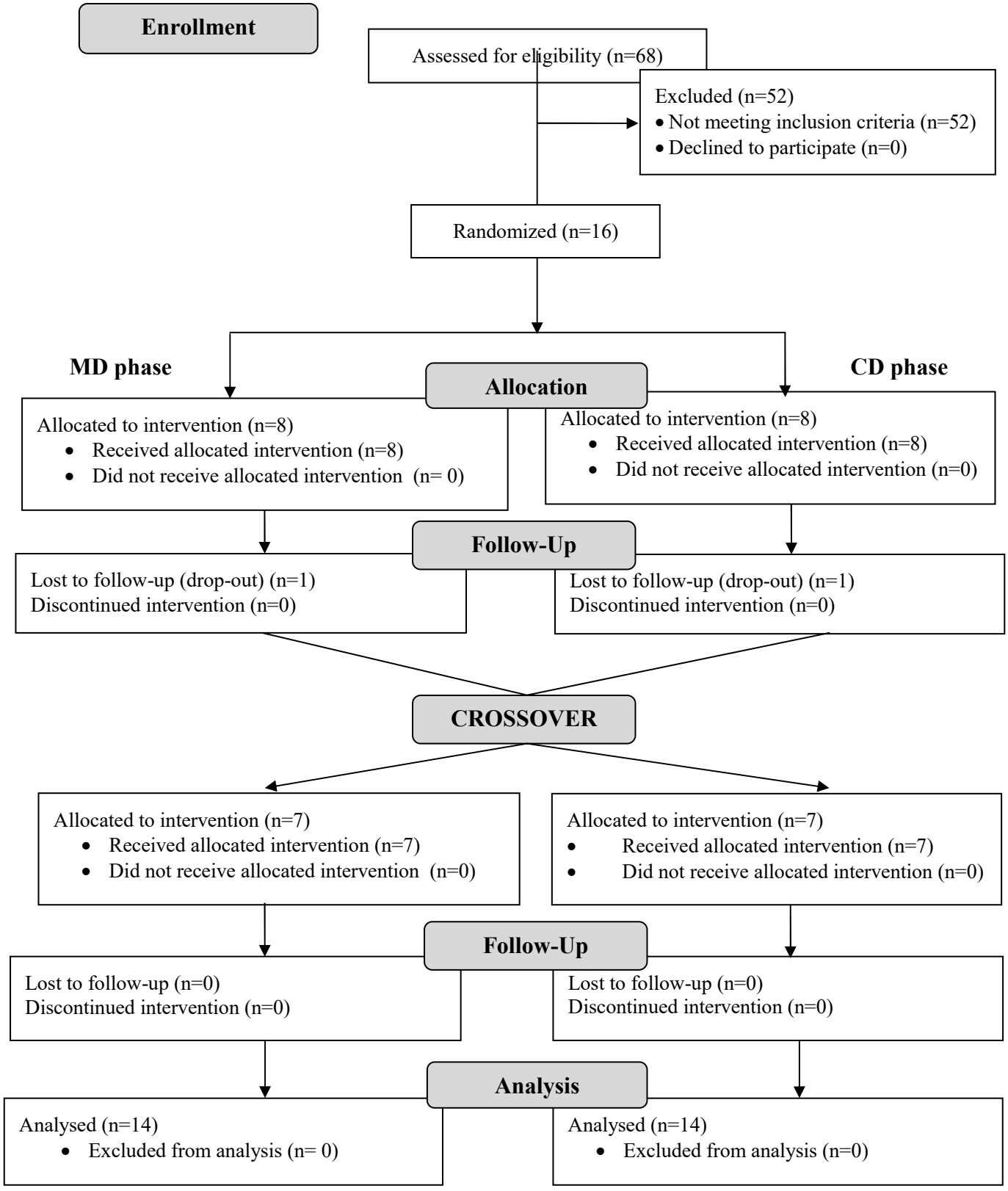

Figure 2 – Subjects flow throughout the study. MD, Fat-free milk phase; CD, Control phase.

4. Discussion

To our knowledge, this is the first study to investigate the effect of the increased consumption of Ca from fat-free milk on MetS components and cardiometabolic measures in subjects with T2DM. Our results indicate that the consumption of ~3 servings of fat-free milk and ~1,200 mg of Ca/d (700 mg of Ca/d from breakfast shakes + 500 mg of dietary Ca under free living condition) during 12 weeks decreased some MetS components and cardiometabolic measures, such as WC, SBP, DBP and LAP index compared to a low-Ca energy restricted diet. Possible mechanisms that may explain the effects of increased fat-free milk consumption on MetS components and cardiometabolic outcomes, based on our results, are presented on **Figure 3**.

The results of some RCTs also indicate that dietary Ca (>1100 mg/d) from dairy consumption decreased WC in adults (Zemel et al., 2005; Torres et al., 2013), but other authors did not find an inverse association between Ca/dairy intake and central adiposity (Wennersberg et al., 2009; Jones et al., 2013). Although overweight or obese subjects were also included in these clinical trials, the main differences between them were the habitual intake of Ca and the energy consumption (eucaloric or energy-restricted diets) during the studies. When the habitual intake of Ca was >800 mg/d, there was no effect on WC (Wennersberg et al., 2009; Jones et al., 2013). A threshold effect for Ca intake equivalent to ~600–800 mg/d has been proposed (Dougkas et al., 2011), probably because low habitual Ca consumers typically present higher absorption of ingested Ca because they use preferentially transcellular pathway (active transport) instead of paracellular pathway (passive transport) (Kopic and Geibel, 2013). In our study, the participants presented low-Ca consumption (<600 mg/d) at baseline of both phases. A meta-analysis of 14 RCTs in 883 adults indicated that increasing dairy consumption only resulted in higher reduction in WC if energy-restricted diet was adopted compared with the consumption of conventional energy-restricted diets alone (Abargouei et al., 2012). The reduction in WC observed in MD was an important result, since WC is a good marker of visceral fat accumulation and is useful for predicting cardiovascular disease (Zemel et al., 2005).

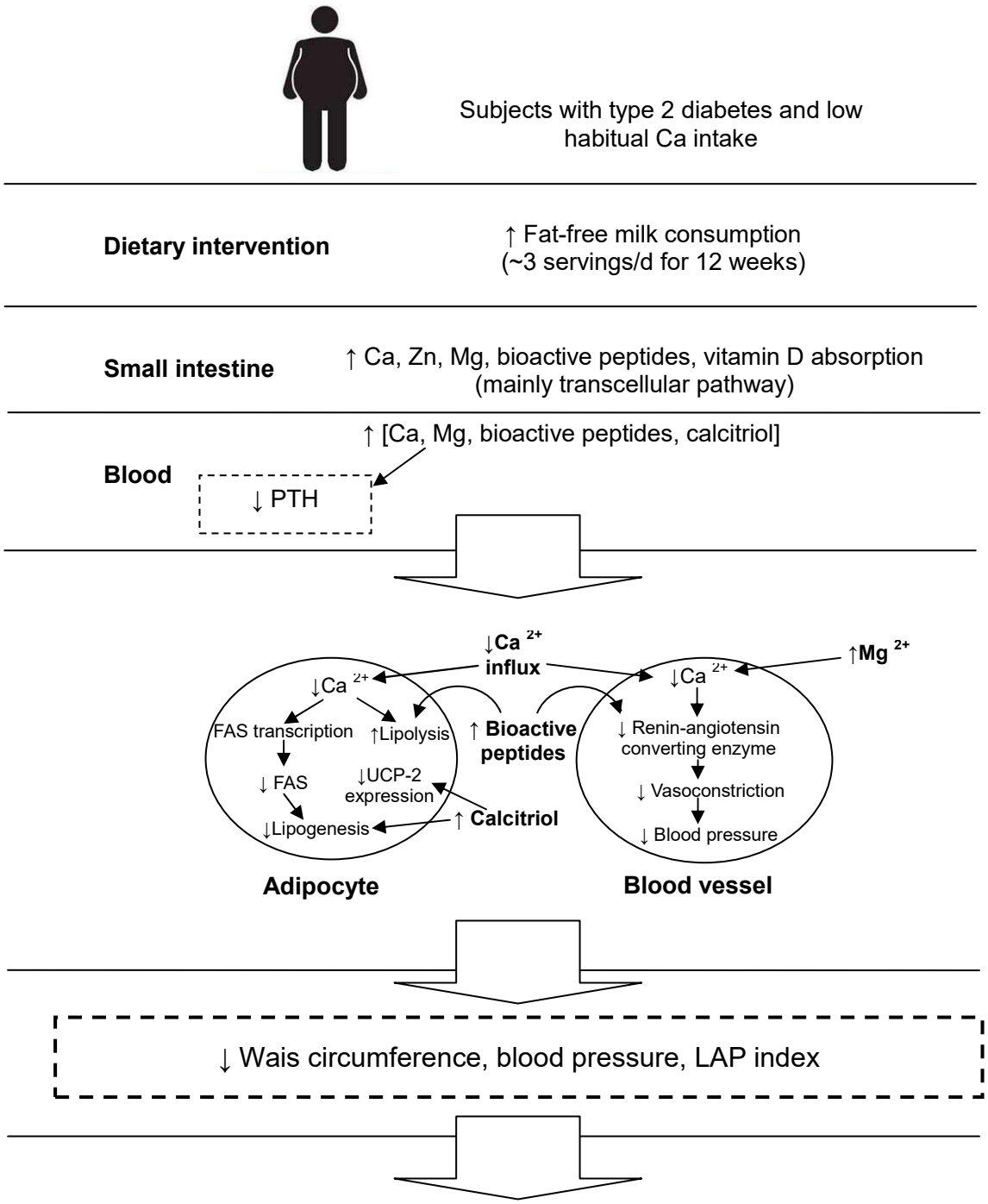

Figure 3 – Possible mechanisms that explain the effects of increased fat-free milk consumption on metabolic syndrome components and cardiometabolic outcomes, based on our results. Ca, calcium; FAS, fatty acid synthase; LAP: lipid accumulation product; Mg, magnesium; PTH: parathormone; UCP-2, uncoupling protein 2.

MD phase also promoted greater reduction of SBP and DBP compared with the CD one. Some RCTs indicated lower BP in response to dairy intervention (Wang et al., 2008; van Meij et al., 2011), while in other studies the increased consumption of dairy foods did not affect BP (van Mierlo et al., 2006; Jones et al., 2013). Crichton et al. (2012) suggest that the effects of dairy consumption on BP are clearly demonstrated only in people with high BP (SBP > 130 mmHg and/or DBP > 85 mmHg). Low-fat dairy products and Ca supplementation equivalent to 1,200 mg/d also contribute to reduce BP, especially in people with habitual Ca intakes <800 mg/d (van Mierlo et al., 2006; Ralston et al., 2012). In our study, we offered fat-free milk, the subjects were low habitual Ca consumers, and their baseline BP before increasing dairy consumption was slightly high (mean SBP 136.6 mmHg, and mean DBP 84.2 mmHg). So we observed a significant reduction of 16 mmHg in SBP and 10.8 mmHg in DBP. Furthermore, the improved BP control with fat-free milk consumption may be clinically relevant, since people with diabetes are more susceptible to cardiovascular diseases (ADA, 2016).

In our study, LAP index was lower after the MD compared with CD. That index has been shown to predict incident T2DM and MetS, and it is considered a better index than BMI in predicting cardiovascular risk (Kahn, 2005), and T2DM (Kahn, 2006). The LAP index is also associated with glucose tolerance (Wehr et al., 2011), so the lower LAP index after high-Ca diets could probably benefit diabetes control. The reduction on WC and on LAP index in MD suggests that this experimental phase promoted visceral adiposity reduction.

Beneficial effects of dairy product intake on MetS components and cardiovascular outcomes can be attributed to its Ca content (Wang et al., 2008; Tong et al., 2011), but also to proteins (Dugan and Fernandez, 2014) and other minerals present in dairy products (Rice et al., 2013). Dietary fat binds to Ca in the intestine and inhibits its absorption, favoring body weight loss (Jacobsen et al., 2005). However, the main effect of high-Ca diets is the decrease in calcitriol (1,25-dihydroxyvitamin D) and PTH concentrations, resulting in lower Ca influx into adipocytes, favoring the occurrence of lipolysis. On the other hand, low-Ca diets increase calcitriol and PTH concentrations, resulting in Ca^{2+} influx into adipocytes. Increased intracellular Ca^{2+} activates lipogenesis and suppresses

lipolysis, increasing body fat and inducing insulin resistance (Zemel et al., 2000). Calcitriol also inhibits the expression of adipocyte uncoupling protein 2 (UCP-2), reducing mitochondrial fatty acid transport and fat oxidation (Zemel et al., 2009). Therefore, the effects observed in this study may have been due to synergic dairy composition.

Ca intake may also interfere in BP. The increased Ca^{2+} influx into smooth muscle cells due to low-Ca consumption can cause vasoconstriction and result in increased BP. So, high-Ca diets seem to prevent Ca-mediated hypertension (Resnick, 1999; Dugan and Fernandez, 2014). Disturbances in intracellular ions, such as increased Ca and decreased Mg, which are common responses in low-Ca diets, lead to vasoconstriction, increased platelet aggregation and thrombosis, and insulin resistance (Resnick, 1999). In combination, Mg acts as a Ca antagonist on smooth muscle tone, blocking Ca^{2+} channels, and thus reducing vasoconstriction (Bo and Pisu, 2008). Milk protein content may also be responsible for its antihypertensive and antiobesity effects. Milk protein inhibits the angiotensin-converting enzyme, consequently reducing BP (Dugan and Fernandez, 2014). In our study, besides the higher Ca content of MD breakfast shakes, both casein and whey protein (MD phase) may have contributed to lower SBP and DBP. On the other hand, the low-Ca and whey protein consumption (CD phase) did not affect BP. Since hydrolyzed casein is less absorbed (Dugan and Fernandez, 2014) and the Ca content of micellar casein (contains 70% of Ca in milk) could not be isolated (Gaucheron, 2005), we offered only whey protein to CD to match the Ca content of the breakfast shakes. However, it is difficult to distinguish which dairy component (Ca or whey protein) has the better effect on MetS components and cardiovascular risk, since dairy products are good sources of the above mentioned nutrients.

A strength of our study was the randomized, controlled crossover design to assess the effects of high-Ca from fat-free milk, hence it reduces between subjects variability. We also controlled PAL and medication use throughout the study. Possible limitations of our study include MD dietary fiber intake increase (~9.4 g/d higher than CD). However, we believe that this difference probably did not influence our results since it has been shown that a daily dietary fiber consumption of ~40 g is necessary to improve glycemia (Silva et al., 2013) and

the consumption of 10g/d of fiber is not enough to increase weight loss (Du et al., 2010).

In conclusion, the consumption of about 3 servings of fat-free milk (700 mg of dietary additional Ca/d) for 12 weeks promoted greater decrease in WC, BP, and LAP index than the control diet in adults with T2DM.

4. Acknowledgments

We thank Instituto Federal do Sudeste de Minas (IF Sudeste MG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Bioclin®, Quibasa Química Ltda.

5. Author Contributions

None of the authors had any personal or financial conflict of interest related to this study. The authors' contributions were as follows: J.M.G.G. designed the study, analyzed and interpreted the data, and wrote the manuscript. J.A.C. assisted in analyzing the data. R.C.G.A. designed the study, interpreted the data, and edited the manuscript. All authors read and approved the final manuscript.

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5.3. ARTICLE 3

Dietary calcium from dairy, body composition and glycemic control in patients with type 2 diabetes: a parallel group randomized clinical trial

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None of the authors had any personal or financial conflict of interest related to this study.

Abstract

Dietary dairy calcium (Ca) seems to favor weight loss and glycemic control, but there is no consensus about that. In this parallel group randomized clinical trial we evaluated the effect of increasing Ca intake on body composition and insulin resistance in patients with type 2 diabetes (T2DM). Thirty-six subjects (48.7 ± 8.9 years old and body mass index 30.7 ± 4.5 kg/m^2), with low habitual Ca intake (<600 mg/d), consumed low-Ca diet (CD group, 800 mg/d) or high-Ca diet (MD group, 1500 mg/d). MD group final anthropometric measures (body weight, BMI, WC, waist-hip ratio, and fat mass) decreased compared to baseline. MD group showed greater decrease in waist circumference compared to CD group. Fasting glucose decreased in CD group. Both groups reduced glycated hemoglobin. Consumption of high-Ca diet for 12 weeks was effective in reducing abdominal adiposity, but provided no additional effect on glycemic control in overweight patients with T2DM.

1. Introduction

Type 2 diabetes *mellitus* (T2DM) is a worldwide public health problem, causing high social and economic costs due to its chronic nature and to the severity of its complications (ADA, 2016). Insulin resistance (IR) characterizes this metabolic disorder and there is usually a relative (rather than absolute) insulin deficiency (ADA, 2016). Therefore, the assessment of insulin sensitivity and β-cell function are useful to identify metabolic abnormalities related to T2DM (Ghasemi et al., 2015).

In overweight patients with T2DM, there are evidences supporting the importance of dietary intervention in order to prevent the occurrence of metabolic abnormalities (ADA, 2016). Although the traditional dietary intervention does not emphasize the role of micronutrients, dietary Ca seems to improve weight loss and glycemic control in T2DM subjects (Abargouei, Janghorbani, Salehi-Marzijarani, Esmaillzadeh, 2012; Soares, Murhadi, Kurpad, Ping-Delfos, & Piers, 2012). However, daily Ca intake is low (~500 mg/d) in industrialized countries, due to the increased consumption of processed foods and reduced consumption of dairy (Imamura et al., 2015).

On the other hand, there is no consensus among authors concerning the effects of Ca supplementation with or without vitamin D, on adiposity, glucose homeostasis and insulin sensitivity (Ferreira, Torres, & Sanjuliani, 2013; Jones et al., 2013; Nikooyeh et al., 2011; Stancliffe, Thorpe, & Zemel, 2011; Torres, Francischetti, Genelhu, & Sanjuliani, 2010). The beneficial effects of increased Ca intake appear to be most significant in low habitual Ca consumers (less than 600 mg of Ca/d) (Zemel et al., 2009), receiving energy-restricted diet (Abargouei et al., 2012) and Ca bioavailable supplements such as citrate or fat-free dairy products (Freitas, Martino, Ribeiro, & Alfenas, 2012; Soares, Ping-Delfos, & Ghanbari, 2011). Besides, few studies assessed the influence of dietary Ca on glycemic control in T2DM subjects, and most (Pittas et al., 2006; Pittas, Lau, Hu, & Dawson-Hughes, 2007; van Dam, Hu, Rosenberg, Krishnan, & Palmer, 2009; Villegas et al., 2009) but not all of them (Nikooyeh et al., 2011; Tabesh, Azadbakht, Faghihimani, Tabesh, & Esmaillzadeh, 2014) were observational. Therefore, considering the potential beneficial effects of adequate Ca intake on glycemic control and the few data regarding the usefulness of dietary Ca on T2DM treatment, this study aimed to evaluate the

effect of increased Ca intake from dairy on body composition and IR in overweight T2DM subjects.

2. Participants and Methods

Described on pages 70-84, “PARTICIPANTS AND METHODS”.

3. Results and Discussion

This study was designed to determine whether a high-Ca intervention could be effective on promoting glycemic control in subjects with T2DM. There is a critical gap in the literature since only a few clinical trials involved T2DM subjects.

A total of 68 subjects were contacted and 40 were eligible according to the inclusion and exclusion criteria. Two from them refused to participate. Thirty-eight subjects were included in the study and two dropped out after enrollment due personal reasons (one from each group). Thirty-six subjects completed the study (number of men/woman was 6/12 and 9/9 in MD and CD groups, respectively). Mean age was 48.9 $\pm$ 9.6 years for MD group and 48.4 $\pm$ 8.3 years for CD group. This study had a power of 80% to detect a reduction of 1% in HbA1c, considering a standard deviation of 2.2%. Anthropometric and body composition measurements, biochemical variables, and food intake did not differ between groups at baseline (**Table 2**).

Ca intake increased after the MD intervention compared to baseline and to the CD group (P<0.01). Body weight, BMI, and WHR decreased in the MD group (P<0.05), but not in the CD group (P>0.05). WC and FM decreased, while FFM increased in both groups after 12-week intervention compared to baseline. Anthropometric and body composition measurements did not differ between groups at the end of the study (P>0.05) (**Table 2**). There was a greater reduction in the MD group WC over 12 weeks from the baseline (delta) compared to CD group (P=0.04) (**Table 3**). Our results suggest that moderate energy restriction associated with Ca intake of ~1200 mg/d during 12 weeks enhanced central adiposity reduction (assessed by WC measurement) in overweight subjects with T2DM. Body weight, BMI, and FM decrease, and FFM increase were similar between groups, which suggest redistribution of body fat in the MD group. This is a relevant result, since central adiposity has been

associated to beta-cell failure and inflammation, exacerbating T2DM micro and macrovascular complications (Laakso & Kuusisto, 2014).

Table 2 - Anthropometric, body composition, biochemical and food intake data at baseline and after 12-week intervention, according to study group.

Characteristics	Baseline MD group (N=18)	Final MD group (N=18)	Baseline CD group (N=18)	Final CD group (N=18)
Anthropometry and body composition measurements				
Body Weight (kg)	80.8±15.6	76.1±16.2*	85.6±17.1	80.7±24.6
BMI (kg/m^2)	30.4±4.7	28.6±4*	31.0±4.9	30.4±4.8
Waist Circumference (cm)	98.3±10.8	93.5±11*	101±11.2	97.9±10.6*
Waist-Hip Ratio (WHR)	0.98±0.06	0.95±0.07*	0.99±0.05	0.97±0.06
Fat Mass (%)	38.8±7.8	37.2±8.5*	36.5±9.1	35.4±8.8*
Fat-Free Mass (%)	59.1±7.5	60.5±8.1*	61.2±8.4	62.2±8.1*
Biochemical variables				
Triglycerides (mg/dL)	175.6±97.6	150.2±80.1	134.2±71.1	123.2±57.2
Fructosamine (µmol/L)	310.5±80.4	300.2±94.5	324.3±89.6	315.8±77.8
Hb1Ac (%)	7.6±1.9	7.2±2.4*	8.3±2.5	7.7±2.1*
Fasting Insulin (µUI/mL)	9.4±3.8	8.9±4.6	7.8±3.8	8.8±5.0
Fasting Glycemia (mg/dL)	145.1±54.1	143.2±67.0	163.6±79.0	148.0±56.3*
HOMA-IR	3.4±1.8	3.1±1.7	2.9±1.9	2.9±1.6
HOMA2-IR	1.4±0.5	1.3±0.6	1.2±0.6	1.3±0.7
TyG index	2.2±0.2	2.1±0.2	2.1±0.2	2.1±0.1
Food Intake				
Energy(kcal/d)	1867.1±530.5	1759.2±427.5	1989.7±471.7	1890.9±340.9
Carbohydrates (g/d)	251.5±92.1	244.2±58.4	259±66	220.1±28.6
Proteins (g/d)	69.2±18.8	82.3±19*	69.5±20.9	82.7±22.7
Total Fat (g/d)	66.4±27.2	48±16.9	66.4±27.1	56.5±38.9
Calcium (mg/d)	414.6±202.4	1193.5±146.1*	462.2±157.1	478.8±201.1

MD group: High-calcium group; CD group: Low-calcium group; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; TyG index: triglyceride-glucose index.

Data are mean ± standard deviation. Baseline characteristics did not differ between groups. *: intra-group statistical difference (P<0.05); **: intergroup statistical difference in the final moment (P<0.05). Baseline and final (12-week intervention) data were analyzed using paired t-test or Wilcoxon test. Between groups data were analyzed using Student t test or Mann-Whitney test.

Table 3 - Anthropometric, body composition, and biochemical data changes from baseline (deltas), according to study group.

Anthropometric and body composition measurements	MD group (N=18)		CD group (N=18)	
	Mean	**DP**	**Mean**	**DP**
Body Weight (kg)	-4.6	7	-4.8	14.1
BMI (kg/m^2)	-1.8	2.9	-1.9	5,7
Waist Circumference (cm)	-4.8	2.7	-3.1	1.9*
Waist-Hip Ratio (WHR)	-0.03	0.02	-0.02	0.02
Fat Mass (%)	-1.6	6.6	-1.1	1.7
Fat-Free Mass (%)	1.4	6.4	1.1	1.8
Biochemical Variables				
Triglycerides (mg/dL)	-25.4	75.9	-10.9	56.3
Fructosamine (µmol/L)	-7.7	35.4	-8.5	49.5
Hb1Ac (%)	-0.4	1.4	-0.6	0.8
Fasting Insulin (µUI/mL)	-0.4	4.3	1.0	5.5
Fasting Glycemia (mg/dL)	-1.9	34.3	-15.6	33.5
HOMA-IR	-0.2	1.2	0.0	2.2
HOMA-IR 2	-0.1	0.5	0.0	0.8
TyG index	-0.1	0.5	0.0	0.1

MD group: High-calcium group; CD group: Low-calcium group; Hb1Ac: glycated hemoglobin; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance. TyG index: triglyceride-glucose index.

* P<0.05. P-value was estimated by paired t-test or Wilcoxon rank sum (both with Bonferroni correction for multiple comparisons).

Similar results were observed in another randomized parallel clinical trial involving 39 obese subjects, which consumed an energy restricted diet (-800 kcal/d) containing high-Ca (1200-1300 mg/d) compared to low-Ca diet (<500 mg/d), during 16 weeks (Torres et al., 2010). Weight loss and glycemic control were similar in both groups, but the high-Ca diet promoted greater abdominal fat reduction (assessed through WC measurement) (Torres et al., 2010). The mechanism suggested for this reduction in central adiposity is not completely clear, though a reduction in cortisol production has been suggested. High-Ca diets suppress 1,25-dihydroxyvitamin D production, which increases 11 beta-hydroxysteroid dehydrogenase type 1, an enzyme that converts inactive cortisone to active cortisol (Soares et al., 2012). Consequently, the decrease in cortisol production from adipose tissue may contribute to the preferential loss of visceral adiposity by high-Ca diets (Soares et al., 2012).

Similar weight loss was observed in overweight subjects (N=49) who received energy restricted diets (-500 kcal/d) with low (700 mg/d) or high-Ca content (1400 mg/d) for 12 weeks, in a randomized parallel clinical trial (Jones et al., 2013). Although weight loss did not differ between experimental groups, high-Ca diet modestly reduced appetite, suggesting the occurrence of a long-term beneficial effect (Jones et al., 2013). No changes in WC were observed in both groups, possibly due to high habitual Ca intake in the control group (943 ± 127 mg/d) and in the dairy/Ca group (884 ± 82 mg/d) (Jones et al., 2013). Zemel et al. (2009) indicate that the benefits of increased dietary Ca consumption has been observed only in low habitual Ca consumers (<700 mg/d).

HbA1c decreased in both groups (P=0.007 for MD group and CD group). Fasting glucose decreased only in the CD group (P=0.05) after 12 weeks of intervention (**Table 2**). Other biochemical parameters and their deltas (changes from baseline) did not differ from baseline and between groups (P>0.05) (**Table 2 and Table 3**). Contrary to our results, some authors observed greater glycemic control and/or decrease in IR after the consumption of high-Ca diets (Nikooyeh et al., 2011; Stancliffe et al., 2011). In metabolic syndrome obese subjects (N=40), the consumption of 3.5 portions/d of whole dairy products (~1000 mg of Ca/d) reduced fasting insulin levels compared to the consumption of 0.5 portion/d of whole dairy products (~150 mg of Ca/d) , during

12 weeks (Stancliffe et al., 2011). Insulin sensitivity (measured by the HOMA-IR) increased after the high-dairy consumption (Stancliffe et al., 2011). In our study, HOMA-IR remained unchanged in both groups. We believe that the differences in these results are due to the characteristics presented by subjects at baseline. Stancliffe et al. (2011) selected subjects with metabolic syndrome and normal glycemia. Our subjects presented hyperglycemia at baseline, and therefore, were probably less responsive to high-Ca diet due their worse metabolic condition. In that study, high-dairy consumption also reduced oxidative stress and inflammation (Stancliffe et al., 2011), parameters that were not assessed in our study.

Other authors observed reductions in Hb1Ac and fasting glycemia in overweight subjects with T2DM (N=30) who received vitamin D– (500 UI of vitamin D), or vitamin D and Ca– (500 UI of vitamin D and 150 mg of Ca) fortified yogurt drink, or placebo (no vitamin D and 150 mg of Ca) (Nikooyeh et al., 2011). Daily intake of a vitamin D–fortified yogurt drink, either with or without added Ca, improved glycemic status in patients with diabetes. That result suggests that dietary Ca was not capable to improve glycemic control (Nikooyeh et al., 2011). However, it is difficult to isolate the vitamin D effects, considering that vitamin D is crucial in regulating Ca absorption in the small intestine (Kopic & Geibel, 2013). Besides, 35% of the subjects were vitamin D deficient at baseline (Nikooyeh et al., 2011), and Ca intake did not differ between groups at baseline and after interventions (Nikooyeh et al., 2011). In our study, fasting glucose decreased only in the CD group, while HbA1c reduced in both groups. HbA1c has greater preanalytical stability, greater association with cardiovascular diseases and T2DM complications, and less day-to-day perturbations than fasting glucose (ADA, 2016). So, in our study high- and low-Ca diets promoted similar improvements on glycemic control. Considering that WC reduction favors an enhancement on insulin sensitivity (ADA, 2016; Soares et al., 2011, 2012), it is possible that the reduction in central fat observed in our study could improve the glycemic profile after a longer period.

A potential mechanism by which high-Ca intake improves glycemic control is its effects on weight loss and adiposity reduction. Increased calcitriol produced in response to low-Ca diets stimulates Ca^{2+} influx in human

adipocytes and thereby promotes adiposity (Soares et al., 2011, 2012). On the other hand, an adequate Ca intake may indirectly enhance calcitriol status, reducing the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D (Kopic & Geibel, 2013). Vitamin D enhances insulin sensitivity and improves β-cell function (Kopic & Geibel, 2013). Ca is regulator of glucose-induced insulin release and it plays a critical role in muscle contraction (Gilon, Chae, Rutter, & Ravier, 2014). That mineral also modulates the interaction of insulin with its receptor, increasing insulin sensitivity (Gilon et al., 2014).

The main limitation of our study is that we did not assess visceral and subcutaneous fat contents, which could confirm Ca effect on body fat redistribution. It is also possible that other components of fat-free milk contributed to a synergistic effect after the consumption of MD diet. Milk proteins and bioactive peptides seem to increase satiety, thermogenesis, and lean mass loss (Acheson et al., 2011; Soares et al., 2012). Leucine favors fat oxidation, and recovery of muscle protein synthesis, thus avoiding FFM loss (Longland, Oikawa, Mitchell, Devries, & Phillips, 2016). That amino acid has also been associated with greater insulin sensitivity and lower oxidative stress (Hirahatake, Slavin, Maki, & Adams., 2014). Other milk minerals such as Zn and Mg play a key role on insulin action. Zn is involved in the synthesis, storage and secretion of insulin, as well as maintaining the insulin hexameric conformational form integrity (Yahya, Yahya, & Saqib, 2011). Mg is essential for insulin secretion and it acts as a cofactor of various enzymes involved in carbohydrates metabolism (Yahya, Yahya, & Saqib, 2011).

5. Conclusion

The consumption of ernergy-restricted diet with high-Ca compared to low-Ca content derived from fat-free milk (~1,200 mg/d) during 12 weeks was more effective in reducing abdominal fat in subjects with T2DM. Dietary intervention improved glycemic control, independently of Ca content. The findings from this study are novel, and have potentially important clinical implications, since few previous clinical trials assessed Ca supplementation in patients with T2DM. However, long-term studies involving subjects with T2DM are needed to better understand the effects of high-Ca intake on glycemic profile, since the reduction in central adiposity may favor insulin sensitivity.

6. Acknowledgements

We thank Instituto Federal do Sudeste de Minas (IF Sudeste MG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Bioclin®, Quibasa Química Ltda. The authors' contributions were as follows: J.M.G.G. and J.A.C. designed the study, analyzed and interpreted the data, and wrote the manuscript. R.C.G.A. designed the study, interpreted the data, and edited the manuscript. All authors read and approved the final manuscript.

7. References

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6. FINAL CONSIDERATIONS

Dietary Ca seems to improve several metabolic disorders related to T2DM control. The literature review suggested some mechanisms by which Ca can modulate the microbiota and intestinal integrity, mainly favoring the growth of beneficial bacteria (i.e. some lactobacilli), reducing the cytotoxicity in the colon. In our review study we verified that subjects with diabetes had higher serum LPS concentrations. Therefore, the effects of dietary Ca on intestinal modulation may favor the reduction of metabolic endotoxemia in subjects with T2DM.

The consumption of approximately 3 servings of fat-free dairy (700 mg of additional Ca, totalizing 1200 mg of Ca/d) compared to the control diet (~ 500 mg of Ca/d) for 12 weeks enhanced weight loss, reduced waist circumference, improved body composition, and reduced cardiovascular risk (mainly decreased blood pressure, total cholesterol, and LAP index) in adults with T2DM and low habitual Ca intake. The effects on glycemic control were more evidenced in the crossover studies compared to the parallel designed one. In general, increased fat-free milk consumption seems to minimize or to delay diabetes complications, and it may be a useful strategy in the dietary treatment of T2DM. In our next study we are planning to investigate the effects of dietary Ca or dairy in the intestinal permeability, gut microbiota, and endotoxemia of individuals with T2DM, since this type of effects have not been investigated yet.

7. APPENDIX 1

Universidade Federal de Viçosa
Centro de Ciências Biológicas e da Saúde
Departamento de Nutrição e Saúde

Nutrição

TERMO DE CONSENTIMENTO LIVRE ESCLARECIDO

"Concordo voluntariamente em participar desse projeto de pesquisa "*Efeito do aumento da ingestão de cálcio na permeabilidade e microbiota intestinais em diabéticos do tipo 2 com excesso de peso*" que tem como finalidade avaliar o efeito de do aumento da ingestão de cálcio na permeabilidade e microbiota intestinais em indivíduos portadores de diabetes tipo 2 com excesso de peso, com duração de 12 semanas. Outras pesquisas demonstram os benefícios do consumo de cálcio na lipólise, resistência insulínica e controle glicêmico. Por isso, este estudo se justifica pela perspectiva de avaliar se o cálcio interfere na permeabilidade e microbiota intestinais, intermediando os efeitos benéficos do cálcio na prevenção e tratamento do diabetes *mellitus*. Durante o estudo, participarei de reuniões semanais. Quinzenalmente, serão agendadas consultas quando haverá avaliação do peso corporal, da composição corporal (com uso de Absorciometria com Raios-X de Dupla Energia - DEXA) e da ingestão alimentar. Nessas consultas, serão prescritas dietas individuais e receberei aconselhamento dietético. Ao início e ao fim do estudo, responderei a questionários e serei submetido a exames para avaliação da microbiota, da permeabilidade intestinal e dos parâmetros bioquímicos (glicemia de jejum, paratormônio, 25 dihidroxi-vitamina D, insulinemia; e níveis de frutosamina, hemoglobina, hemoglobina glicada (HbA1c), albumina glicada, ácido úrico, triglicérideos, colesterol total e frações, lipopolissacarídeo, interleucina 6, fator de necrose tumoral, proteína C reativa e adiponectina). Poderei ser alocado em grupo experimentar ou controle, de acordo com o sorteio realizado pelos pesquisadores. Terei que entregar amostras de fezes e urina, além de coletar amostras de sangue por punção venosa durante o experimento. No momento da coleta de sangue, poderá haver sensação incômoda e pequenos hematomas poderão aparecer no dia seguinte à coleta, em decorrência da punção da pele. Para minimizar estes riscos, a coleta sanguínea será realizada por profissional de enfermagem habilitado para a realização desses

procedimentos, sendo as coletas feitas com o paciente em posição confortável. Após a coleta, o local da punção será comprimido durante quatro a seis minutos, evitando a perda de maiores quantidades de sangue. O aparelho DEXA, usado na avaliação da composição corporal dos voluntários, utiliza doses mínimas de radiação, sem risco significativo para a saúde. Não existe risco de reação adversa no procedimento indicado anteriormente, uma vez que para esse tipo de avaliação não se emprega qualquer tipo de medicamento, contraste ou radiotraçador. O exame é indolor e sem procedimentos invasivos (sem uso de agulhas, cateteres, sondas ou incisões). Os alimentos fornecidos durante o estudo não oferecerão risco à saúde dos voluntários, uma vez que os mesmo são normalmente ingeridos pelas pessoas. Receberei como benefício um plano alimentar individual nutricionalmente adequado, visando à adequação da ingestão de cálcio e redução de peso corporal. Além disso, terei acesso aos meus dados das avaliações antropométrica, composição corporal, bioquímica, permeabilidade e microbiota intestinal, presença ou não de componentes da síndrome metabólica e o possível risco de um acidente cardiovascular resultados comigo. Sou sabedor que não receberei nenhum tipo de vantagem econômica ou material por participar do estudo, além de poder abandonar a pesquisa em qualquer etapa de seu desenvolvimento, sem nenhuma penalização. Estou em conformidade que meus resultados obtidos, sejam divulgados no meio científico, sempre resguardando minha privacidade e identificação. Todos os gastos com a pesquisa serão de total responsabilidade dos pesquisadores. Caso tenha algum gasto com a pesquisa, serei ressarcido pelos pesquisadores e receberei indenização diante de eventuais danos decorrentes da pesquisa. Tanto o material biológico quanto os questionários serão armazenados durante o período de 5 (cinco) anos, sob responsabilidade da coordenadora do projeto. Após este período, os questionários serão destruídos (picotados) e o material biológico será descartado conforme orientações da ANVISA. Estou suficientemente informado pelos membros do presente estudo, sobre as condições em que os procedimentos do referido estudo acontecerão, sob responsabilidade da profa. Dra. Rita de Cássia Gonçalves Alfenas e sua equipe de trabalho.”

Eu, _____,
fui informado(a) dos objetivos do presente estudo de maneira clara e detalhada e esclareci minhas dúvidas. Sei que a qualquer momento poderei solicitar novas informações e modificar minha decisão de participar se assim o desejar. Declaro que concordo em participar desse estudo. Recebi uma cópia deste termo de consentimento livre e esclarecido e me foi dada a oportunidade de ler e esclarecer as minhas dúvidas.

Viçosa, ____ de _____ de 20____.

Assinaturas:

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Em caso de dúvidas com respeito aos aspectos éticos deste estudo, você poderá consultar:
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