The Journal of Nutrition

Human milk short-chain fatty acid composition is associated with adiposity outcomes in infants

--Manuscript Draft--

Manuscript Number:	JN-2018-1037R2
Full Title:	Human milk short-chain fatty acid composition is associated with adiposity outcomes in infants
Short Title:	Breast milk short-chain fatty acids and growth
Article Type:	Original Research Article
Section/Category:	Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions
Keywords:	breast milk, short chain fatty acids, lipids, nutrition, weight, growth, adiposity, infancy
Corresponding Author:	David B Dunger, MD University of Cambridge Cambridge, UNITED KINGDOM
Corresponding Author's Institution:	University of Cambridge
First Author:	Philippa M Prentice, Dr
Order of Authors:	Philippa M Prentice, Dr
	Marieke H Schoemaker, Dr
	Jacques Vervoort, Professor
	Kasper Hettinga, Professor
	Tim T Lambers, Dr
	Eric AF van Tol, Dr
	Carlo L Acerini, Dr
	Laurentya Olga, Dr
	Clive J Petry, Dr
	Ieuan A Hughes, Professor
	Albert Koulman, Dr
	Ken K Ong, Professor
	David B Dunger, Professor
Abstract:	Background: Presumed benefits of human breast milk (HM) in avoiding rapid infancy weight gain and later obesity could relate to its nutrient composition. However, data on breast milk composition and its relationship with growth are sparse. Objective: We investigated whether short-chain fatty acids (SCFAs), known to be present in HM and linked to energy metabolism, are associated with infancy anthropometrics. Methods: In a prospective birth cohort, HM hind milk samples were collected from 619 lactating mothers at 4-8 weeks postnatally [median(IQR) age: 33.9(31.3-36.5) y, BMI (kg/m2): 22.8(20.9-25.2)]. Their offspring, born at 40.1(39.1-41.0) weeks gestation with weight: 3.56(3.22-3.87) kg and 51% male, were assessed with measurement of infant weight, length, and skinfold thicknesses at ages 3, 12, and 24 months, and transformed to age and sex-adjusted z-scores. HM SCFAs were measured by 1H-nuclear magnetic resonance spectroscopy (NMR) and gas chromatography (GC-MS). Multivariable linear regression models were conducted to analyse the relationships between NMR HM SCFAs and infancy growth parameters with adjustment for potential confounders. Results: NMR peaks for HM butyrate, acetate, formic acid, but not propionate, were detected. Butyrate peaks were 17.8% higher in HM from exclusively breastfeeding mothers than mixed-feeding mothers (p=0.003). HM butyrate peak-values were

	negatively associated with changes in infant weight (standardized B=-0.10, p=0.019) and BMI (B=-0.10, p=0.018) between 3 and 12 months, and negatively associated with BMI (B=-0.10, p=0.018) and mean skinfolds thickness (B=-0.10, p=0.049) at age 12 months. HM formic acid peak-values showed a consistent negative association with infant BMI at all time points (B<=-0.10, P<=0.014) while HM acetate was negatively associated with skinfolds thickness at 3 months (B=-0.10, p=0.028) and 24 months (B=-0.10, p=0.036). Conclusions: These results suggest HM SCFAs play a beneficial role in weight gain and adiposity during infancy. Further knowledge of HM SCFAs function may inform future strategies to support healthy growth.
Response to Reviewers:	
Additional Information:	
Question	**Response**
Please select a collection option from the list below:	Obesity and Metabolism Research Articles
Author Comments:	Wednesday 12th December 2018 Teresa A. Davis, Ph.D. Editor-in-Chief Journal of Nutrition Dear Dr. Davis, We have thoroughly revised our manuscript following the reviewers' helpful comments, and believe that it is now an improvement over the previous version. We hope that it is now considered suitable for publication. Thank you for your consideration. With best wishes, David Dunger MD F Med Sci Corresponding author Professor Emeritus and Director of Research NIHR Senior Investigator Emeritus Box 116 Level 8 Cambridge Biomedical Campus Cambridge CB2 0QQ Tel: +44 (0) 1223 336886 Fax: +44 (0) 1223 336996 Email: dbd25@cam.ac.uk http://paediatrics.medschl.cam.ac.uk/

Human milk short-chain fatty acid composition is associated with adiposity outcomes in infants

Author Names

Philippa M Prentice[1]

Marieke H Schoemaker[2]

Jacques Vervoort[3]

Kasper Hettinga[3]

Tim T Lambers[2]

Eric AF van Tol[2]

Carlo L Acerini[1]

Laurentya Olga[1]

Clive J Petry[1]

Ieuan A Hughes[1]

Albert Koulman[4]

Ken K Ong[1, 4]

David B Dunger[1]

Author Affiliations

1. Department of Paediatrics, Wellcome Trust-MRC Institute of Metabolic Science, NIHR Cambridge Comprehensive Biomedical Research Centre, University of Cambridge, Cambridge, UK
2. Mead Johnson Pediatric Nutrition Institute, Nijmegen, the Netherlands
3. Wageningen University, the Netherlands
4. MRC Epidemiology Unit, Wellcome Trust-MRC Institute of Metabolic Science, University of Cambridge, UK

Corresponding author: Professor David B Dunger, Department of Paediatrics Box 116, University of Cambridge, Cambridge Biomedical Campus, Cambridge CB2 0QQ

Tel: +44 1223 336886 Fax: +44 1223 336996 Email: dbd25@cam.ac.uk

Authors' last names: Prentice, Schoemaker, Vervoort, Hettinga, Lambers, van Tol, Acerini, Olga, Petry, Hughes, Koulman, Ong, Dunger

Word count (Introduction-Conclusion): 2,737 words

Number of figures: 2

Number of tables: 2

Running title

Breast milk short-chain fatty acids and growth

Abbreviations

BMI	Body mass index
CBGS	Cambridge Baby Growth Study
CIs	Confidence intervals
GC-MS	Gas Chromatography
HM	Human milk
IQR	Interquartile range
mo	Month
^{1}H-NMR	^{1}H-Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
SCFAs	Short Chain Fatty Acids
SDS	Standard deviation score
TGs	Triglycerides
WHO	World Health Organization

Sources of support: PP was supported by a Medical Research Council Clinical Training Fellowship (G1001995). The Cambridge Baby Growth Study has been supported by the European Union (QLK4-1999-01422), the World Cancer Research Foundation International (2004/03), the Medical Research Council (7500001180), the NIHR Cambridge Comprehensive Biomedical Research Centre, Newlife - The Charity for Disabled Children (07/20), Mothercare Foundation (RG54608), and Mead Johnson Nutrition. KKO is supported by the Medical Research Council (MC_UU_12015/2).

Conflict of Interest Statement: This study received unconditional funding support from Mead Johnson Nutrition. MHS, TTL and EAFT are employees of Mead Johnson Nutrition. No other authors declare a conflict of interest.

Abstract

Background: Presumed benefits of human milk (HM) in avoiding rapid infancy weight gain and later obesity could relate to its nutrient composition. However, data on breast milk composition and its relationship with growth are sparse.

Objective: We investigated whether short-chain fatty acids (SCFAs), known to be present in HM and linked to energy metabolism, are associated with infancy anthropometrics.

Methods: In a prospective birth cohort, HM hind milk samples were collected from 619 lactating mothers at 4-8 weeks postnatally [median(IQR) age: 33.9(31.3-36.5) y, BMI (kg/m^2): 22.8(20.9-25.2)]. Their offspring, born at 40.1(39.1-41.0) weeks gestation with weight: 3.56(3.22-3.87) kg and 51% male, were assessed with measurement of weight, length, and skinfolds thickness at ages 3, 12, and 24 months, and transformed to age and sex-adjusted z-scores. HM SCFAs were measured by ^{1}H-nuclear magnetic resonance spectroscopy (NMR) and gas chromatography (GC-MS). Multivariable linear regression models were conducted to analyze the relationships between NMR HM SCFAs and infancy growth parameters with adjustment for potential confounders.

Results: NMR peaks for HM butyrate, acetate, formic acid, but not propionate, were detected. Butyrate peaks were 17.8% higher in HM from exclusively breastfeeding mothers than mixed-feeding mothers (p=0.003). HM butyrate peak-values were negatively associated with changes in infant weight (standardized B=-0.10, p=0.019) and BMI (B=-0.10, p=0.018) between 3 and 12 months, and negatively associated with BMI (B=-0.10,

p=0.018) and mean skinfolds thickness (B=-0.10, p=0.049) at age 12 months. HM formic acid peak-values showed a consistent negative association with infant BMI at all time points (B<=-0.10, P<=0.014) while HM acetate was negatively associated with skinfolds thickness at 3 months (B=-0.10, p=0.028) and 24 months (B=-0.10, p=0.036).

Conclusions: These results suggest HM SCFAs play a beneficial role in weight gain and adiposity during infancy. Further knowledge of HM SCFAs function may inform future strategies to support healthy growth.

Keywords: breast milk, short chain fatty acids, lipids, nutrition, weight, growth

Introduction

Early postnatal nutrition is critical for infant optimal growth and associated with long-term health outcomes[1]. The type of infant milk feeding, as well as specific dietary compositions and volume of intake, may be important factors.

Human milk (HM) intake has been associated with beneficial immunological responses, infancy growth patterns and potential later obesity risk reduction[2–4]. A diverse range of bioactive components in HM could contribute to these protective effects and may be associated with different patterns of weight and adiposity gain during infancy[5]. Nevertheless, data on HM composition and relationships with growth are limited.

Fat (triglycerides and fatty acids) is an important nutritional constituent of HM. It derives either from the maternal circulation or is synthesized in the mammary glands[6–8]. We recently reported that the fat % energy in HM is inversely associated with subsequent weight gain and adiposity during infancy, suggesting functional implications of HM macronutrient contents[9]. The quality and quantity of fat and fatty acid constituents, such as short-chain fatty acids (SCFAs: acetate, butyrate, formate, propionate, and valerate) could be relevant to infant growth. SCFAs as well as being a constituent of foods, are synthesized in the gut by an anaerobic microbiota during fermentation of HM oligosaccharides. Butyrate is frequently measured in faeces, but this is only a small proportion of what is being produced in the gut. Its circulating concentration is affected by liver and intestinal epithelial cell metabolism, intestinal absorption, and systemic distribution[10,11]. Butyrate

is present in significant quantities in bovine milk[11], however, its origin, form, concentration, and biological function in HM deserves further study[12]. Butyrate, a 4-C fatty acid, has previously been detected in HM[13,14], but it is unclear if it is esterified to triglycerides (TGs) or exists as free butyrate. SCFAs are highly volatile and such data are limited by a lack of consensus on assay methods, HM concentrations, and functional relevance.

Preclinical studies show that prebiotics and/or SCFAs intake is associated with lower body weight, and suggest that SCFAs may have a complex role in energy metabolism. For example, acetate intake has been associated with lower body weight in animals[15–21]. In high-fat fed mice, butyrate intake reportedly attenuates obesity-associated inflammation and insulin resistance[22], possibly by activating the SCFAs receptor, GPR43[23].

Here, we aimed to confirm the presence of SCFAs in HM and test the hypothesis that HM SCFAs concentrations are associated with growth and adiposity during infancy.

Subjects and Methods

Study Design: The Cambridge Baby Growth Study (CBGS: 2001-2009) is a prospective birth cohort, set up to investigate antenatal and early postnatal determinants of infancy growth, as described previously[24]. In brief, women were recruited in early pregnancy from a single hospital in Cambridge, UK, and followed through pregnancy and postnatally. The cohort included 1585 singleton, late preterm or full-term born (gestational age ≥ 36 weeks) infants with measurements at birth; 64% of mothers exclusively breastfed their infants at 8 weeks postnatally. This analysis is based on a subset of 619 mother-infant dyads, where a HM sample was collected. All mothers gave informed written consent and the study was approved by the Cambridge local research ethics committee.

Anthropometry: Trained nurses measured infant weight, length and skinfolds thickness in the first 8 days of life, and then at ages 3, 12, and 24 months. Weight was measured to the nearest 1g (Seca 757 electronic baby scale). Supine length was measured to the nearest 0.1cm (Infantometer SECA 416), and BMI was then calculated. Skinfolds thickness were measured in triplicate at 4 body sites (triceps, subscapular, flank, quadriceps) on the left side of the body (Tanner/Whitehouse Skinfold Caliper, Holtain Ltd).

HM collection: Women were asked to hand express hindmilk into low binding glass bottles, after feeding their infant, from the breast that they had last used for feeding. In order to reduce within-day and day-to-day variations and allow for comparable samples, this collection was repeated multiple times

over a two week period between 4-8 weeks postnatally in order to collect a total pooled sample of 100 mL hindmilk. HM samples were kept frozen to prevent volatilization of SCFAs. All samples were processed together at a single time point, with the oldest samples being stored continuously at -20^{0}C for 16 years. Each pooled HM sample was mixed thoroughly before analysis. Infant feeding (exclusive breast- vs. mixed-feeding) was reported by questionnaire at 3 months of age, with questions about current feeding, and age at starting infant formula milk. Infants were categorized as either exclusively breast- or mixed-fed at 8 weeks of age, at the time of HM sampling.

HM assays: SCFAs concentrations were measured in homogenized HM samples using ^{1}H-Nuclear magnetic resonance (NMR) spectra and Gas chromatography (GC-MS).

^{1}H-NMR analyses: To determine SCFAs composition with NMR, 400 microliters (µL) of a homogenized HM sample was mixed with 400 µL CDC_3 solvent for 10 minutes and then centrifuged [Eppendorf centrifuge 5424 (Eppendorf AG, Hamburg, Germany)] for 30 minutes at 9,500 *g*. The polar fraction was then used to measure total SCFAs content as NMR spectra peak heights as described by Prentice et al[9].

To determine the contribution of HM TGs to total butyrate NMR peak-value, HM samples were exposed to lipase (Candida rugose, Sigma L8525, lyophilized powder, ≥40,000 units/mg protein). Per 100 µL HM, 16 µL Lipase

stock (3 mg/mL) was added and incubated for 17 hours at 37^0C. After cooling to 0^0C, samples were prepared for NMR. One sample was spiked with tributyrin (Sigma T8626) as a positive control.

GC-MS free butyrate measurements: One mL HM samples were preheated in 10 mL vials sealed with silicon/Teflon septa and magnetic caps for 1 minute at 60^0C. Volatile metabolites were extracted from the headspace for 5 minutes with a 75 µm PDMS-carboxen SPME fibre (Supelco, Bellefonte, PA, USA) using the combiPAL autosampler (CTC Analytics AG, Switzerland). The volatile metabolites were thermally desorbed from the fibre by heating it in a Best PTV injector with an empty liner for 5 min at 250^0C. The fibre was subsequently cleaned for 10 min at 290^0C. A vial with air was used as blank. GC separation of the volatile components was performed on a Finnigan Trace GC gas chromatograph (ThermoFinnigan, San Jose, CA, USA) coupled to a Finnigan DSQ mass spectrometer (ThermoFinnigan, San Jose, CA, USA), using a polar Stabilwax-DA column of 30 m length, 0.32 mm i.d., and 0.32 mm film thickness (Restek Bellefonte, PA, USA). The oven temperature was held at 40^0C for 2 minutes, raised to 220^0C at 15^0C/minutes, followed by 1 minute holding. Helium was used as the carrier gas at a flow rate of 1.5 mL/minute (Stabilwax-DA column). The MS interface and the ion source were kept at 250^0C. Acquisition was performed in electron impact mode (70 eV) with 2 scans/s; the mass range used was *m/z* 33-250.

GC-MS chromatograms were analyzed for peak identification using AMDIS software (NIST, Gaithersburg, MD). Identification of volatile metabolites was

based on matching mass spectra and retention time with pure standards. Volatile metabolites were integrated using the XCalibur software package (ThermoFinnigan, San Jose, CA, USA). The peak area (corrected for blank if necessary) in arbitrary units was used for statistical analysis. Absolute quantification was undertaken using standard addition of butyrate to milk samples.

Anthropometry variables: Age and sex-appropriate standard deviation scores (SDS) were calculated for infant weight, length, and BMI, adjusting for gestational age at birth, by comparison to the UK 1990 growth reference[25] using LMS Pro software[26]. An internal SDS was calculated for each of the four skinfolds thickness, adjusting for age, and the mean of the four skinfolds thickness SDS was used as an estimate of total adiposity.

Statistics: NMR SCFAs concentrations were square-root transformed to normalize their distributions; the SCFAs content was calculated from the sum of the individual NMR SCFAs peak-values; GC-MS butyrate concentrations were similarly log-transformed. Spearman rank-correlation was used to assess the relationship between the NMR (total) butyrate peak concentrations with the GC-MS (free) butyrate concentrations. Relationships between NMR HM SCFAs and infancy growth parameters were tested in multivariable linear regression models, including the following covariates: birth weight, gestational age, infant sex, infant milk feeding, and HM storage time. To assess associations between HM butyrate peak-values and infant growth that were independent of HM TGs concentration, the latter variable

was entered as an additional covariate to the regression models. Therefore, we used a residual nutrient method[9]: HM butyrate peak-values were regressed against HM TGs concentrations, and the resulting standardized residuals were tested for association with infant growth. Analyses were performed using SPSS version 21, and statistical significance indicated by p-value <0.05.

Results

As many as 619 mothers of singleton, term or late preterm infants provided a HM sample (453 exclusively breastfeeding at 8 weeks postnatally, 124 mixed-feeding, 42 unknown feeding type). There was no difference between this subgroup and all breastfeeding mother-infant pairs in the whole CBGS cohort with respect to gestational age, maternal age, pre-pregnancy BMI, primiparity, ethnicity, infant size at birth or growth to 12 months of age[9]. (**Table 1**).

HM SCFAs content

For the 619 HM samples collected at age 4-8 weeks, NMR peaks were detected for butyrate, acetate, and formic acid, but not propionate (**Table 2**). HM volatile phase free butyrate was also detected by GC-MS (N=102), at concentrations ranging from 0 to 3.5 mg per 100 ml. NMR (total) butyrate peak concentrations were moderately well correlated with GC-MS (free) butyrate concentrations (Spearman Rho, r_s=0.53, p<0.0005, **Figure 1**).

Modest inter-correlations were detected between NMR HM SCFAs peak-values. Butyrate peak-values were weakly positively correlated with acetate peak-values (r_s=0.10, p=0.010) and weakly negatively correlated with formic acid peak-values (r_s= -0.09, p=0.021); acetate was weakly positively correlated with formic acid (r_s=0.10, p=0.016). HM total fat (TGs) concentrations were positively correlated with NMR butyrate peak-values (r_s=0.67, p<0.0005; Figure 1) and were weakly positively correlated with acetate peak-values (r_s=0.12, p=0.003), but were not correlated with formic acid peak-values (r_s=0.00, p=0.9).

Lipase treatment did not change NMR butyrate peak-values in CBGS HM samples but increased these peak-values in positive control HM samples spiked with tributyrin (*data not shown*), indicating that degradation of TGs in HM samples post-collection does not contribute to total butyrate content.

Correlates of HM SCFAs content

HM SCFAs content (butyrate, acetate, or formic acid) was not associated with maternal BMI, parity, mode of delivery, socioeconomic status, gestational age, infant birth weight or sex. HM butyrate peak-values modestly declined with longer HM storage time (r_s= -0.13, p=0.001), whereas acetate and formic acid peak-values increased. Butyrate peak-values were higher in HM from exclusively breastfeeding mothers compared to those mixed-feeding: median (IQR) peak-value 2.81 (2.07) vs. 2.31 (2.11) (p=0.001), but acetate and formic acid peak-values did not differ (not shown). Subsequent analyses were adjusted for HM storage time and infant feeding group.

HM SCFAs associations with infancy growth

There were no associations between any SCFAs content and infant size at birth, or with infant length at any age. HM (NMR) butyrate peak concentrations were negatively associated with changes in infant weight (standardized B=-0.10, p=0.019) and BMI (B=-0.10, p=0.018) between ages 3 to 12 months, and negatively associated with BMI (B=-0.10, p=0.018) and mean skinfolds thickness (B=-0.10, p=0.049) at age 12 months (**Table 2**). These apparent effects on infant adiposity were attenuated by age 24 months as a consequence of positive associations between HM butyrate peak-values and changes in infant BMI and skinfolds thickness between 12 to 24 months (Table 2).

HM formic acid showed a consistent negative association with infant BMI at all time points (3, 12 and 24 months: all B<=-0.10, P<=0.014) while HM acetate was negatively associated with skinfolds thickness at ages 3 months (B=-0.10, p=0.028) and 24 months (B=-0.10, p=0.036) (Table 2). Adjustments for HM TGs concentrations attenuated the above associations with HM butyrate peak concentrations, but the associations with HM formic acid and HM acetate largely persisted (Table 2).

Discussion

This study provides evidence that the SCFAs, butyrate, formic acid, and acetate, are detectable in HM and show largely negative associations with measures of infant adiposity. These effects were most prominent between

age 3-12 months and were predictably less evident when mixed feeding had been introduced by age 12-24 months. Thus our data provide preliminary data that breast milk SCFAs concentrations may provide some early protection against excess weight gain. We accept that this interpretation is based on the assumption that lower weight gain and reduced fat accretion is beneficial but this is the prevailing assumption concerning the protective effects of breast milk feeding on the risk of obesity. These associations with infancy growth have not been previously reported, likely due to the lack of large mother-infant cohort studies with HM samples and limitations in SCFAs measurements.

However, our observations are supported by experimental data from animal models that identified a role for SCFAs including butyrate, in regulating lipid metabolism and body weight gain. Dietary butyrate supplementation reversed high-fat diet-induced weight gain and detrimental changes in fat tissue and metabolic outcomes[27], such as obesity-associated inflammation and insulin resistance[22,28]. The resistance to obesity development by butyrate supplementation may be explained in part by increased thermogenesis and energy expenditure[16] resulting from activation of brown adipose tissue through increased sympathetic activity[29].

In addition to oral dietary butyrate administration, the endogenous production of SCFAs resulting from microbial carbohydrate fermentation has been shown to affect peripheral tissues, by acting as signal transduction molecules through G-protein coupled receptor (GPCR) interaction, and as epigenetic

277 modulators of gene expression via inhibition of histone deacetylase
278 (HDAC)[21].
279
280 In adipose tissue, butyrate affects adipogenesis and fat cell metabolism,
281 either directly or indirectly through anti-inflammatory mechanisms. This would
282 suggest a role for butyrate in metabolic processes, healthy weight
283 development, and mitigating inflammation in adipose tissue, thereby
284 potentially reducing the risk of obesity[30,31]. The observation that butyrate acts
285 as an epigenetic regulator[32] may indicate a role for HM derived butyrate in
286 the epigenetic regulation of adipose tissue function, hence affecting the
287 programming of healthy weight development in offspring. Alternatively it has
288 been proposed that acetate may have a direct role in regulating central
289 appetite regulation[33].
290
291 Thus, although the exact mechanisms remain to be elucidated, in addition to
292 endogenously produced butyrate, HM derived butyrate may contribute to the
293 regulation of adiposity and its related metabolic changes. Based on the
294 results from the current study HM butyrate may stimulate normal infant
295 adiposity and metabolism, thus supporting healthy weight development in
296 early life. Interestingly directly after birth, endogenous SCFAs and butyrate
297 production in the infant gut is relatively low which gradually increases over
298 time while the microbiota matures[10]. It is thus interestingly to speculate that
299 HM may provide an initial supply of butyrate to the newborn while
300 endogenous production by its immature microbiota is low.
301

The exact source of HM butyrate remains to be elucidated. Our current findings suggest that HM butyrate exists largely as a free SCFA, as the addition of enzymatic lipase to samples ex vivo did not increase the HM butyrate peaks. However, we cannot exclude the contribution of glycerides as lipases in HM are expressed in significant amounts and contribute to lipid digestion and overall fatty acid bioavailability in the developing infant[34]. Furthermore, HM butyrate could derive from synthesis by microbiota, which is known to be resident in HM[35].

Unfortunately, HM volume intakes were not assessed in our study and therefore we cannot assess whether the associations observed with HM SCFAs contents were mediated by SCFAs rather than total intakes. Mothers were asked to pool, over a period of 2 weeks, their collected expressed hind milk samples and information on the timings of milk collection was not recorded. Future studies should include measurements of HM intakes and assessment of the feasibility of using lipase inhibitors to reduce hydrolysis of TGs to better understand the origin of SCFAs. The observation that butyrate levels were higher in the milk of mothers who exclusively breastfed is interesting but unexplained although it may relate to the length of feeds as we know TGs levels are increased in hind milk. Repeated longitudinal HM collections and assessment of the microbiome will provide data necessary to understand the relationships between HM SCFAs and infant growth. Such understanding may help to elucidate the potential beneficial properties of HM in supporting optimal growth and adiposity during infancy, particularly in settings where rapid weight gain and obesity are common.

Conclusion: HM SCFAs, mainly butyrate measured as free acid in hind milk, may play a beneficial role in weight gain and adiposity during infancy. Further knowledge of HM and exploration of HM SCFAs origin may inform future strategies to support healthy growth.

Acknowledgements: We acknowledge the CBGS research nurses Suzanne Smith, Anne-Marie Wardell & Karen Forbes. We thank all the families who contributed to the study, the staff at the Cambridge NIHR/Wellcome Trust Clinical Research Facility, the NIHR Cambridge Comprehensive Biomedical Research Centre, and the midwives at the Rosie Maternity Hospital, Cambridge, UK. We acknowledge Kelly Dingess for her support with butyrate measurements.

Authors' contributions:

The authors' responsibilities were as follows: PMP, CLA, KKO, DBD: designed the project; PMP, JV, KH, AK: conducted the research; PMP, MHS, JV, KKO: performed statistical analyses; PMP, MHS, TTL, AK, LO, CP, JV, EAFT, KKO, DBD: wrote the manuscript; DBD had primary responsibility for final content; and all authors: critically revised the manuscript for important intellectual content, read and approved the final manuscript.

References

1. Lanigan J, Singhal A. Early nutrition and long-term health: A practical approach. *Proc Nutr Soc*. 2009;68:422-9. doi:10.1017/S002966510999019X.
2. Ziegler EE. Growth of Breast-Fed and Formula-Fed Infants. *Nestlé Nutr Work Ser Pediatr Progr*. 2006;58:51-63.
3. Stein AD, Barros FC, Bhargava SK, Hao W, Horta BL, Lee N, Kuzawa CW, Martorell R, Ramji S, Stein A, et al. Birth status, child growth, and adult outcomes in low- and middle-income countries. *J Pediatr*. 2013;163:1740-6.e4. doi:10.1016/j.jpeds.2013.08.012.
4. Ong KK, Emmett P, Northstone K, Golding J, Rogers I, Ness AR, Wells JC, Dunger DB. Infancy weight gain predicts childhood body fat and age at menarche in girls. *J Clin Endocrinol Metab*. 2009;94:1527-32. doi:10.1210/jc.2008-2489.
5. Ballard, O. Morrow A. Human milk composition: nutrients and bioactive factors. *Pediatr Clin North Am*. 2013;60:49-74. doi:10.1016/j.pcl.2012.10.002.
6. Jensen RG, Lammi-Keefe CJ, Ferris AM, Jackson MB, Couch SC, Capacchione CM, Ahn HS, Murtaugh M. Human milk total lipid and cholesterol are dependent on interval of sampling during 24 hours. *J Pediatr Gastroenterol Nutr*. 1995;20:91-4.
7. Hamosh M, Bitman J. Human milk in disease: Lipid Composition. *Lipids*. 1992;27:848-57. doi:10.1007/BF02535863.
8. Hester SN, Hustead DS, MacKey AD, Singhal A, Marriage BJ. Is the macronutrient intake of formula-fed infants greater than breast-fed infants in early infancy? *J Nutr Metab*. 2012;2012. doi:10.1155/2012/891201.
9. Prentice P, Ong KK, Schoemaker MH, van Tol EAF, Vervoort J, Hughes IA, Acerini CL, Dunger DB. Breast milk nutrient content and infancy growth. *Acta Paediatr*. 2016;105:641-7. doi:10.1111/apa.13362.
10. Verbeke KA, Boobis AR, Chiodini A, Edwards CA, Franck A, Kleerebezem M, Nauta A, Raes J, van Tol EAF, Tuohy KM. Towards microbial fermentation metabolites as markers for health benefits of prebiotics. *Nutr Res Rev*. 2015;28:42-66.

doi:10.1017/S0954422415000037.

11. McNabney SM, Henagan TM. Short chain fatty acids in the colon and peripheral tissues: A focus on butyrate, colon cancer, obesity and insulin resistance. *Nutrients*. 2017;9:1-28. doi:10.3390/nu9121348.
12. Walker WA, Shuba Iyengar R. Breastmilk, microbiota and intestinal immune homeostasis. *Pediatr Res*. 2014;77:1-9. doi:10.1038/pr.2014.160.
13. Precht D, Molkentin J. C18:1, C18:2 trans and cis fatty acid isomers including conjugated cis Δ9, trans Δ11 linoleic acid (CLA) as well as total fat composition of German human milk lipids. *Nahrung*. 1999;43:233-44.
14. Li G, Yao W, Jiang H. Short-chain fatty acids enhance adipocyte differentiation in the stromal vascular fraction of porcine adipose tissue. *J Nutr*. 2014;144:1887-95. doi:10.3945/jn.114.198531.
15. Roy CC, Kien CL, Bouthillier L, Levy E. Short-chain fatty acids: ready for prime time? *Nutr Clin Pract*. 2006;21:351-66. doi:10.1177/0115426506021004351.
16. Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M, Cefalu WT, Ye J. Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes*. 2009;58:1509-17. doi:10.2337/db08-1637.
17. Lin HV, Frassetto A, Kowalik EJ, Nawrocki AR, Lu MM, Kosinski JR, Hubert JA, Szeto D, Yao X, Forrest G, et al. Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS One*. 2012;7:1-9. doi:10.1371/journal.pone.0035240.
18. Carvalho BM, Jose M, Saad A. Influence of gut microbiota on subclinical inflammation and insulin resistance. *Mediators Inflamm*. 2013;2013:1-13.
19. Yadav H, Lee JH, Lloyd J, Walter P, Rane SG. Beneficial metabolic effects of a probiotic via butyrate-induced GLP-1 hormone secretion. *J Biol Chem*. 2013;288:25088-97. doi:10.1074/jbc.M113.452516.
20. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host

energy metabolism. *J Lipid Res*. 2013;54:2325-40. doi:10.1194/jlr.R036012.

21. Kasubuchi M, Hasegawa S, Hiramatsu T, Ichimura A, Kimura I. Dietary gut microbial metabolites, short-chain fatty acids, and host metabolic regulation. *Nutrients*. 2015;7:2839-49. doi:10.3390/nu7042839.
22. Vinolo MAR, Rodrigues HG, Festuccia WT, Crisma AR, Alves VS, Martins AR, Amaral CL, Fiamoncini J, Hirabara SM, Sato FT, et al. Tributyrin attenuates obesity-associated inflammation and insulin resistance in high-fat-fed mice. *Am J Physiol Endocrinol Metab*. 2012;303:E272-82. doi:10.1152/ajpendo.00053.2012.
23. Kimura I, Inoue D, Hirano K, Tsujimoto G. The SCFA receptor GPR43 and energy metabolism. *Front Endocrinol (Lausanne)*. 2014;5:1-3. doi:10.3389/fendo.2014.00085.
24. Prentice P, Acerini CL, Eleftheriou A, Hughes IA, Ong KK, Dunger DB. Cohort profile: The Cambridge Baby Growth Study (CBGS). *Int J Epidemiol*. 2016;45:35-35g. doi:10.1093/ije/dyv318.
25. Freeman J V., Cole TJ, Chinn S, Jones PRM, White EM, Preece MA. Cross sectional stature and weight reference curves for the UK, 1990. *Arch Dis Child*. 1995;73:17-24. doi:10.1136/adc.73.1.17.
26. Pan H, Cole T. LMSgrowth, a Microsoft Excel add-in to access growth references based on the LMS method. 2012. http://www.healthforallchildren.co.uk/.
27. Arnoldussen IAC, Wiesmann M, Pelgrim CE, Wielemaker EM, van Duyvenvoorde W, Amaral-Santos PL, Verschuren L, Keijser BJF, Heerschap A, Kleemann R, et al. Butyrate restores HFD-induced adaptations in brain function and metabolism in mid-adult obese mice. *Int J Obes*. 2017;41:935-44. doi:10.1038/ijo.2017.52.
28. Raso GM, Simeoli R, Russo R, Iacono A, Santoro A, Paciello O, Ferrante MC, Canani RB, Calignano A, Meli R. Effects of sodium butyrate and its synthetic amide derivative on liver inflammation and glucose tolerance in an animal model of steatosis induced by high fat diet. *PLoS One*. 2013;8:1-13. doi:10.1371/journal.pone.0068626.
29. Li Z, Yi CX, Katiraei S, Kooijman S, Zhou E, Chung CK, Gao Y, van den Heuvel JK, Meijer OC, Berbée JFP, et al. Butyrate reduces appetite and activates brown adipose

tissue via the gut-brain neural circuit. *Gut*. 2018;67:1269-1279. doi:10.1136/gutjnl-2017-314050.

30. Meijer K, De Vos P, Priebe MG. Butyrate and other short-chain fatty acids as modulators of immunity: What relevance for health? *Curr Opin Clin Nutr Metab Care*. 2010;13:715-721. doi:10.1097/MCO.0b013e32833eebe5.
31. Brahe LK, Astrup A, Larsen LH. Is butyrate the link between diet, intestinal microbiota and obesity-related metabolic diseases? *Obes Rev*. 2013;14:950-959. doi:10.1111/obr.12068.
32. Davie J. Inhibition of histone deacetylase activity by butyrate. *J Nutr*. 2003;133:2485S-2493S.
33. Frost G, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, Anastasovska J, Ghourab S, Hankir M, Zhang S, et al. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. *Nat Commun*. 2014;5:1-11. doi:10.1038/ncomms4611.
34. Lindquist S, Hernell O. Lipid digestion and absorption in early life: An update. *Curr Opin Clin Nutr Metab Care*. 2010;13:314-20. doi:10.1097/MCO.0b013e328337bbf0.
35. McGuire MK, McGuire MA. Got bacteria? The astounding, yet not-so-surprising, microbiome of human milk. *Curr Opin Biotechnol*. 2017;44:63-8. doi:10.1016/j.copbio.2016.11.013.

TABLE 1 Participant characteristics in the current analysis (who provided a human milk sample) and wider Cambridge Baby Growth Study cohort[1]

	Current sample: with HM sample	All breast- or mixed-fed
Demographics		
Participants, *n*	619	924
Gestational age, weeks	40.1 (39.1-41.0)	40.0 (39.1-41.0)
Maternal age, years	33.9 (31.1-36.5)	34.0 (31.2-36.5)
Maternal BMI, kg/m^2	22.8 (20.9-25.2)	22.7 (20.8-25.2)
Index of deprivation	9.0 (6.9-9.0)	9.0 (6.8-9.0)
Maternal primiparity, *n*(%)	266(43)	388(42)
White Caucasian, *n*(%)	594(96)	887(96)
Infant male sex, *n*(%)	316(51)	471(51)
Exclusive breast-feeding, *n*(%)	452(73)	712(77)
Infant growth		
Birth		
Weight, kg	3.56 (3.22-3.87)	3.55 (3.22-3.85)
Length, cm	51.5 (50.0-53.5)	51.5 (50.0-53.3)
Mean skinfolds thickness, mm	6.2 (5.3-7.4)	6.1 (5.2-7.3)
BMI, kg/m^2	13.3 (12.2-14.3)	13.3 (12.2-14.2)
3 months		
Weight, kg	6.10 (5.60-6.64)	6.09 (5.59-6.62)
Length, cm	61.2 (59.4-63.0)	61.2 (59.5-63.0)
Mean skinfolds thickness, mm	10.8 (9.4-11.9)	10.7 (9.4-11.9)
BMI, kg/m^2	16.3 (15.4-17.2)	16.2 (15.3-17.2)
12 months		
Weight, kg	9.85 (9.10-10.60)	9.88 (9.15-10.60)
Length, cm	75.8 (74.0-77.7)	75.6 (73.9-77.7)
Mean skinfolds thickness, mm	11.0 (9.8-12.5)	11.0 (9.7-12.4)
BMI, kg/m^2	17.1 (16.2-18.0)	17.1 (16.3-18.1)

[1]Values are medians (IQRs) unless otherwise indicated

TABLE 2 HM SCFAs peak-values measured by NMR and their associations with infant growth from 3-24 mo[1]

Associations with infant growth	*SCFAs* (ranges)					
	Butyrate (0-0.4 mM)		Formate (0.1-7.5 mM)		Acetate (0.1-8.5 mM)	
	B	*P*	B	*P*	B	*P*
Weight SDS						
3 mo	0.03	0.4	-0.04	0.2	-0.04	0.3
12 mo	-0.03	0.4	-0.08	0.054	-0.02	0.6
24 mo	0.03	0.5	-0.12	0.005*	-0.05	0.3
Δ Weight SDS						
3-12 mo	-0.10	0.019	-0.05	0.3	0.02	0.7
12-24 mo	0.09	0.04	-0.03	0.5	-0.05	0.3
BMI SDS						
3 mo	-0.01	0.7	-0.10	0.011[2]	-0.05	0.3
12 mo	-0.10	0.018	-0.11	0.014[2]	0.00	0.9
24 mo	0.02	0.7	-0.12	0.007[2]	0.02	0.6
Δ BMI SDS						
3-12 mo	-0.11	0.010	0.01	0.9	0.04	0.4
12-24 mo	0.13	0.005	0.01	0.8	-0.01	0.8
Mean skinfolds SDS						
3 mo	-0.02	0.7	-0.02	0.6	-0.10	0.028[2]
12 mo	-0.08	0.049	-0.03	0.5	-0.05	0.3
24 mo	0.01	0.8	-0.10	0.021[2]	-0.10	0.036[3]
Δ Skinfolds thickness SDS						
3-12 mo	-0.08	0.057	-0.01	0.9	0.06	0.2
12-24 mo	0.09	0.047	-0.07	0.14	-0.09	0.071

[1]Values are standardized regression coefficients (p-values) adjusted for exclusive breast- vs. mixed feeding at 8 weeks, sex, GA, birthweight, duration of sample storage. SCFA concentrations were square-root transformed.
[2]P<0.05 following additional adjustment for HM TGs concentrations
[3]P=0.055 following additional adjustment for HM TGs concentrations

Figure Legends

Figure 1: Scatterplot of HM butyrate peak values measured by NMR versus concentrations measured by GC-MS (n=102) (A) and HM TGs concentrations (B) (n=619).

GC-MS, gas chromatography; HM, human breast milk; NMR, nuclear magnetic resonance; TGs, triglycerides.

Figure 2: Changes from 3-12 mo in infants' body weight SDS (A), BMI SDS (B), and skinfolds SDS by quintiles of HM butyrate peak values measured by NMR at 4-8 wk of age. Values are group means and 95% CIs.

BMI, body mass index; CIs, confidence intervals; HM, human breast milk; NMR, nuclear magnetic resonance; SDS, standard deviation score.

Figure 1

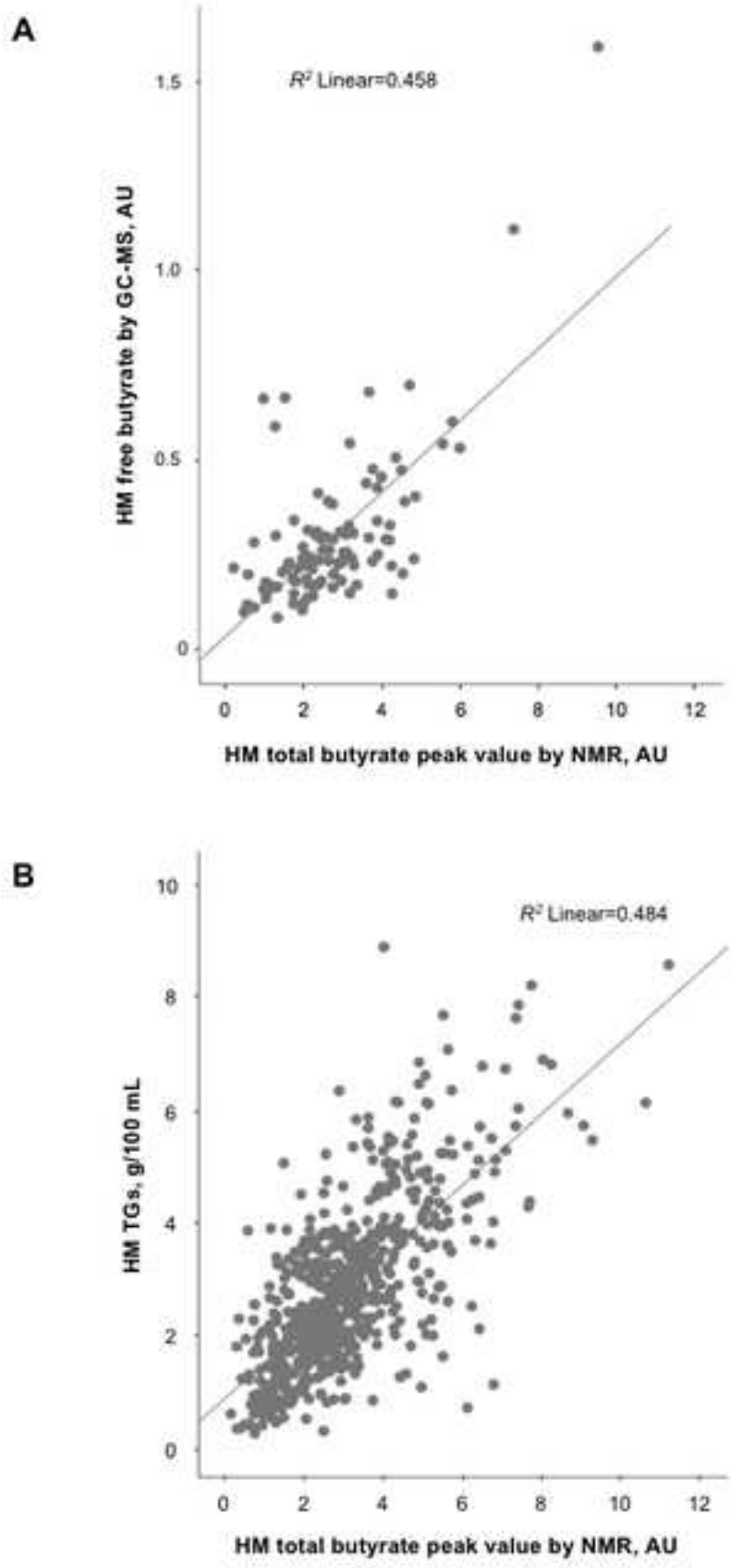
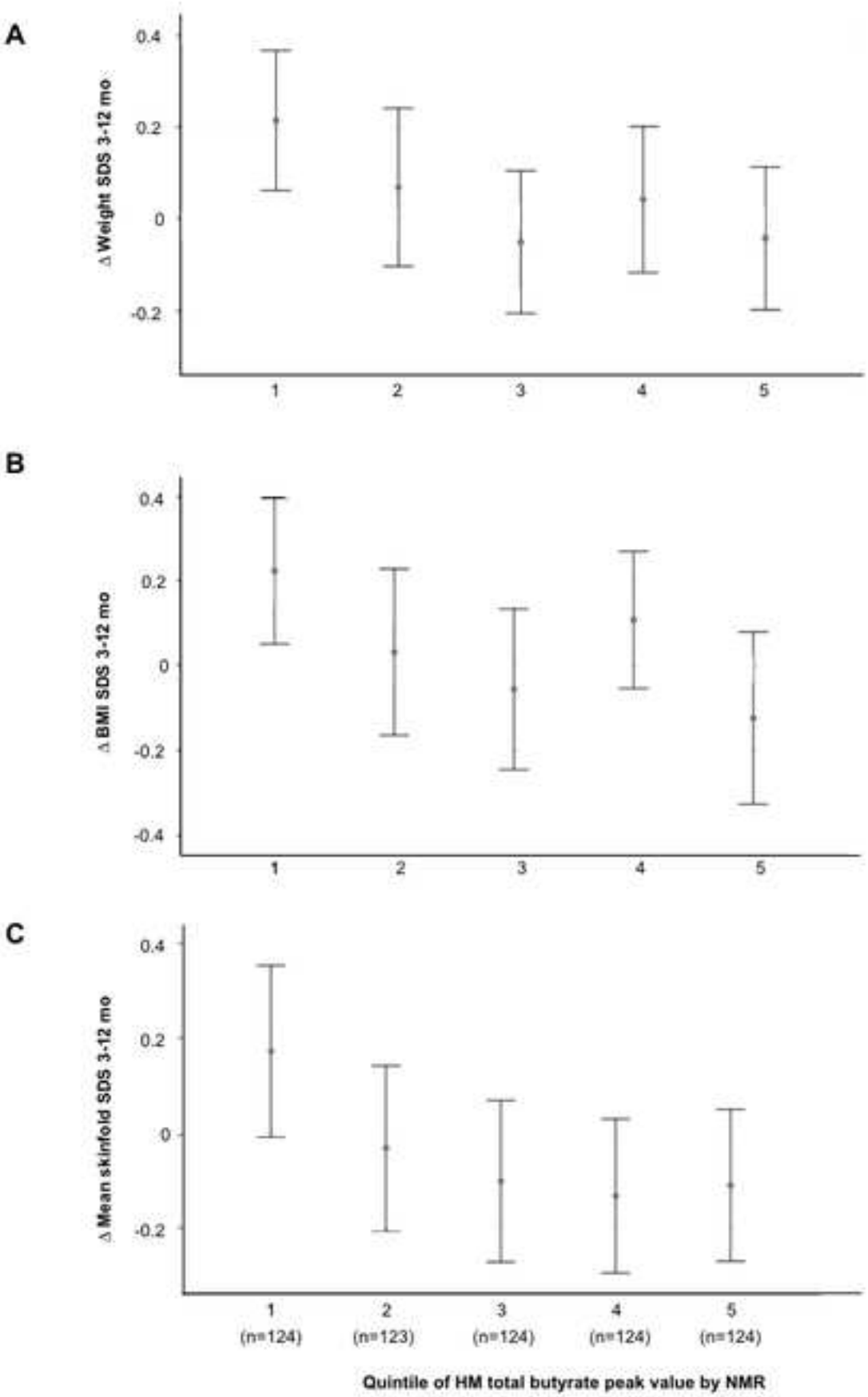


Figure 2



Participant flow chart (as part of the Online Supporting Material)