

## Title

Higher weight and weight gain after 4 years of age rather than weight at birth are associated with adiposity, markers of glucose metabolism and blood pressure in 5-year-old Ethiopian Children

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## **Short running head**

Weight gain and cardiometabolic markers at 5 years

## **Supplementary data**

Supplemental Tables 1-2, Supplemental Figures 1-9, Supplemental Methods and

Supplemental References are available from the "Supplementary data" link in the online

posting of the article and from the same link in the online table of contents at

<https://academic.oup.com/jn/>.

## **Abbreviations**

Body composition (BC), confidence interval (CI), developmental origins of health and disease (DOHaD), fat mass (FM), fat-free mass (FFM), homeostasis model assessment of insulin resistance index (HOMA-IR), infant Anthropometry and Body Composition (iABC), International Wealth Index (IWI), linear-spline mixed effects (LSME), standard deviation score (SDs), World Health Organization (WHO).

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## **Conflict of Interest (COI) Statement**

None of the authors had any conflicts of interest.

## 1 **Abstract**

### 2 *Background*

3 Fetal and early life growth is associated with adult risk of obesity and cardiometabolic  
4 disease. However, little is known about the relative importance of birth weight and  
5 successive periods of weight gain on markers of cardiometabolic risk in childhood in low-  
6 income populations.

### 7 *Objective*

8 The objective was to study associations of birth weight and weight gain velocities in selected  
9 age intervals from birth to 60 mo with height, fat-free mass and markers of adiposity and  
10 cardiometabolic risk at 60 mo.

### 11 *Design*

12 In a prospective cohort study of 375 Ethiopian children aged 60 mo, we estimated individual  
13 weight gain velocities in the periods between birth, 3, 6, 24, 48 and 60 mo using linear-spline  
14 mixed-effects modelling. Subsequently, we analyzed associations of birth weight, weight  
15 gain velocities and current weight with height, fat-free mass and markers of adiposity and  
16 cardiometabolic risk.

### 17 *Results*

18 Weight gain from 48-60 mo and weight at 60 mo rather than birth weight were the strongest  
19 correlates of insulin, C-peptide, HOMA-IR, blood pressure, height, fat-free mass, waist  
20 circumference and fat mass at 60 mo. For instance, 1 SDs higher (1 SDs = 50 g/mo) weight  
21 accretion from 48-60 mo was associated with a higher insulin of 23.3% (95% confidence  
22 interval: 9.6, 38.8), C-peptide of 11.4% (2.7, 20.8), systolic blood pressure of 1.4 mmHg (0.6,

23 2.3), fat mass of 0.72 kg (0.59, 0.85), and fat-free mass of 0.70 kg (0.56, 0.85). Weight gain  
24 from 0-3 mo was positively associated with LDL cholesterol, systolic blood pressure, height  
25 and the BC indices, and weight gain from 24-48 mo was inversely associated with blood  
26 glucose.

### 27 *Conclusions*

28 In 60-mo-old Ethiopian urban children, weight gain and weight after 48 mo rather than  
29 weight at birth may represent a sensitive period for variations in markers of adiposity and  
30 glucose metabolism.

31

## 32 **Clinical Trial Registry**

33 The birth cohort is registered in ISRCTN (<https://www.isrctn.com/>): identifier  
34 ISRCTN46718296.

35

## 36 **Keywords**

37 birth weight; body composition; cardiometabolic status; cohort study; child, preschool;  
38 developmental origins of health and disease; Ethiopia; growth; infant; linear-spline mixed-  
39 effects model.

## 40 **Introduction**

41 Growth patterns in fetal life and early childhood have been associated with risk of obesity  
42 and cardiometabolic disease in adulthood in numerous studies (1-5), and it has been  
43 suggested that the risk of detrimental cardiometabolic adaptations is most pronounced  
44 when poor pre- and postnatal growth are followed by accelerated weight or body mass  
45 index (BMI) gain in childhood (6-10). This is commonly referred to as the developmental  
46 origins of health and disease (DOHaD) hypothesis, where environmental stimuli or insults in  
47 fetal life and early childhood, can cause lasting metabolic alterations in the developing child.  
48 Studies from middle- (11-13) and high-income populations (14-23) have found that  
49 cardiometabolic adaptations related to accelerated growth in early life may be initiated  
50 already in childhood. Thus far, no studies from low-income countries have examined the  
51 relative importance of birth weight and successive periods of weight gain in early life on  
52 subsequent body composition (BC) and markers of cardiometabolic risk in early childhood.  
53 This is surprising, as the combination of persistent high rates of undernutrition, steadily  
54 increasing rates of childhood and adult obesity, and an accelerating nutritional transition are  
55 likely to make these populations particularly vulnerable to the programming effects of  
56 growth in early life (24-26). Furthermore, the effects of variability in early-life growth on  
57 later BC appear to be population-specific (27, 28). Thus, to develop appropriate nutrition-  
58 specific interventions to prevent the emerging epidemic of obesity and cardiometabolic  
59 disease facing many low-income countries in sub-Saharan Africa (29, 30), it is important to  
60 improve our understanding of the timing of how growth in early-life is related to markers of  
61 adiposity and cardiometabolic risk in these settings.

62 Commonly, studies in the DOHaD literature have assessed growth from a single or only a few  
63 measurements such as fetal growth indexed by birth weight or weight change from birth to  
64 two years. With these study designs it is not possible to examine the dynamic weight  
65 changes that occur throughout infancy. Moreover, life-course studies of fetal and early  
66 childhood antecedents of later disease risk often rely on unbalanced data, where repeated  
67 measures within individuals are not measured at exactly the same time points and the  
68 sample size at each time point are changing. Therefore, appropriate analyses require  
69 statistical methods that can flexibly and robustly estimate dynamic changes in early life and  
70 are able to account for the dependencies of repeated measures within the same individual  
71 as well as an unbalanced data structure (31, 32). We modelled changes in weight in early  
72 childhood using Linear-spline Mixed-effects (LSME) modelling which can accommodate these  
73 issues. In this longitudinal study, we therefore aimed to investigate the associations of birth  
74 weight, weight gain velocities in selected age intervals from birth to 60 mo and weight at 60  
75 mo with BC and markers of cardiometabolic status at 60 mo of age in Ethiopian children.

## 76 **Subjects and methods**

### 77 **Study setting and participants**

78 Infants and mothers were enrolled in the infant Anthropometry and Body Composition  
79 (iABC) birth cohort study between December 2008 and October 2012 at Jimma University  
80 Specialized Hospital, Jimma, Ethiopia. All mothers residing in the town of Jimma (estimated  
81 population of 157,432 (33)), giving birth to a term child (gestation >37 weeks) with a birth  
82 weight of  $\geq 1500$  g without congenital malformations were eligible to participate. If informed  
83 consent was obtained, the children were enrolled and examined within 48 hours after  
84 delivery. The study participants were scheduled for 12 visits between birth and 60 mo of age  
85 (0, 1.5, 2.5, 3.5, 4.5, 6, 12, 18, 24, 36, 48, 60 mo). A detailed description of the iABC cohort is  
86 found elsewhere (34, 35).

87

### 88 **Data collection**

#### 89 *Anthropometry from birth to 60 mo*

90 Weight from birth to 6 mo was measured to the nearest 0.1 g using the inbuilt scale of a PEA  
91 POD (COSMED, Rome, Italy), from 12-36 mo to the nearest 0.1 kg using an electronic UNICEF  
92 scale (SECA, Hamburg, Germany) and from 48-60 mo to the nearest 1 g using the inbuilt  
93 scale of a BOD POD (COSMED, Rome, Italy). Height at 60 mo was measured to the nearest  
94 0.1 cm using a SECA 213 portable height measurer (SECA, Hamburg, Germany). Waist  
95 circumference at 60 mo was measured to the nearest 0.1 cm in standing position with feet  
96 together midway between the iliac crest and lowest costal margin using a non-stretchable  
97 measuring tape. The anthropometric measurements were done by two research nurses,  
98 trained according to the standard operating procedures as recommended by the World



99 Health Organization (WHO) (36). Throughout the data collection period, we ran regular  
100 refreshment trainings as well as interobserver monitoring of the anthropometry  
101 assessments to ensure accuracy and reliability.

#### 102 *Body composition at 60 mo*

103 Fat mass (FM) and fat-free mass (FFM) at 60 mo were measured using the BOD POD – an air  
104 displacement plethysmograph with a pediatric chair insert. The BOD POD system is an  
105 accurate, precise, feasible and safe method for assessment of BC in children (37, 38). During  
106 the measurement, the child was placed in a pediatric chair insert in an enclosed test  
107 chamber, not wearing any clothes besides a swim cap and tight fitted underpants. A  
108 complete BOD POD measurement lasted approximately 5-10 minutes. Each morning the  
109 research nurses calibrated both the PEA POD and the BOD POD using standardized weight  
110 and volume cylinders from the manufacturer to ensure accurate and precise assessments of  
111 child weight and volume. All the calculations were performed by the inbuilt computer of the  
112 BOD POD (software version 5.2.0). A detailed description of the theory and calculations  
113 behind the BC method can be found elsewhere (39).

#### 114 *Blood pressure at 60 mo*

115 Systolic and diastolic blood pressure (mmHg) were measured in sitting position after the  
116 child had relaxed for a minimum of 5 minutes. Measurement were done in duplicate using a  
117 blood pressure monitor with age-appropriate cuffs and averaged (Pressostabil model, Welch  
118 Allyn Inc., Skaneateles Falls, USA).

119 *Cardiometabolic markers at 60 mo*

120 A 2 mL venous blood sample was collected from the antecubital fossa by a laboratory  
121 technician. The mother was instructed not to give her child any food or drinks 2 hours prior  
122 to arriving at the clinic, and the blood was sampled as the last element in the assessment  
123 battery, yielding a minimum of 3 hours of fasting. Blood glucose (mmol/L) was determined  
124 on whole blood samples using the HemoCue Glucose System (HemoCue, Ängelholm,  
125 Sweden). Glycosylated hemoglobin (HbA1c, mmol/mol) was measured on whole blood  
126 samples using a DCCT aligned Quo-Test® A1c Analyzer (EKF Diagnostics, Cardiff, Wales).  
127 Subsequently, serum was obtained by centrifuging the whole blood sample, aliquoted in  
128 3x0.4 mL and frozen at -80°C until analyzed. The serum samples were analyzed at the  
129 Ethiopian Public Health Institute, using the module c501 of the COBAS 6000 analyzer (Roche  
130 Diagnostics International Ltd, Rotkreuz, Switzerland) for total-, LDL- and HDL cholesterol and  
131 triglyceride concentrations (all lipids in mmol/L) and the module e601 for insulin ( $\mu\text{U}/\text{mL}$ )  
132 and C-peptide (ng/mL). The homeostasis model assessment of insulin resistance index  
133 (HOMA-IR) was calculated as  $\text{insulin} \times \text{glucose} / 22.5$ .

134 *Covariates*

135 Information on birth order, sex, gestational age, maternal age and educational level, family  
136 socioeconomic status and breastfeeding status was collected through questionnaires, that  
137 were double entered and corrected for any discrepancies. Maternal height was measured to  
138 the nearest 0.1 cm using a Seca 214 Stadiometer (SECA, Hamburg, Germany). Gestational  
139 age was obtained by physical examination of the newborn by trained research nurses using  
140 the New Ballard Score test (40). Family socioeconomic status was estimated using the  
141 International Wealth Index (IWI), a comprehensively tested index of the material well-being

142 of households in low and middle income countries (41, 42). IWI uses information of 12  
143 material well-being dimensions, including 7 household assets, access to 2 public services,  
144 and 3 characteristics of the house to measure the extent to which the basic needs of the  
145 household are met. IWI is measured on a scale from 0 to 100 (highest wealth). Breastfeeding  
146 status at 4 to 6 mo post-partum was divided into 4 categories: exclusive (no other foods  
147 given), almost exclusive (no other foods given except water), predominant (breast milk as  
148 primary food) and partial/none (breast milk not the primary food/not breast feeding) (43).  
149 These covariates were considered potential confounding variables in the linear regression  
150 models.

151

## 152 Ethics

153 Ethical permission was granted from the Jimma University Ethical Review Committee (Ref.  
154 no. RPGC/279/2013). Written informed consent was obtained from parents or caregivers of  
155 all eligible participants. There were no risks related to the examinations, and before the 2 ml  
156 of venous blood was drawn from the child a topical anesthetic (EMLA crème) was applied to  
157 the skin. Children with medical conditions observed by the research nurses during the  
158 examinations were referred according to local clinical guidelines.

159

## 160 Statistical methods

161 Individual weight gain velocities were computed for 5 selected age intervals from birth to 60  
162 mo of age (Step 1), and then associations of birth weight, weight gain velocities and current  
163 weight with BC and cardiometabolic markers at 60 mo were modelled (Step 2).

164 *Estimated birth weight and weight gain velocities from 0-60 mo*

165 To approximate the non-linear relationship between weight and age in children, a linear-  
166 spline mixed effects (LSME) model was applied to estimate the individual weight gain  
167 velocities in 5 pre-specified age intervals from birth to 60 mo (44, 45). The LSME model  
168 builds upon a mixed effects modelling approach by joining together two or more linear  
169 mixed effects functions at the knot points of pre-specified age intervals. Within each age  
170 interval the growth velocity or the slope of the curve is specified to be linear. This allows  
171 children to change growth velocity as they age from birth to 60 mo. Children with at least  
172 one weight measurement at birth and at the 60-mo visit were included in the modelling of  
173 the individual growth velocities. A more technical description of the LSME modelling for this  
174 study is presented in the Supplementary data (**Supplemental Methods**).

175 *Associations of birth weight and weight gain velocities with body composition and*  
176 *cardiometabolic markers*

177 In separate models, the cardiometabolic markers measured at the 60-mo visit were  
178 regressed on estimated birth weight and the weight gain velocities derived in step 1 as well  
179 as on current weight at the 60-mo visit, respectively. Prior to the regression analysis, the  
180 birth weight, weight gain velocities and current weight were standardized to a standard  
181 deviation score (SDs) of 1 and a mean of 0 to obtain comparable model estimates across the  
182 different growth periods. Thus, the beta coefficients from the regression analyses indicate  
183 the change in outcome per study population SDs of the exposure variables (e.g. weight gain  
184 from 0-3 mo). The analyses were adjusted for the following covariates in separate models:  
185 Model 1 was adjusted for sex, birth order and gestational age. Model 2 was additionally  
186 adjusted for the child's exact age at the 60-mo visit, maternal age at delivery, maternal

187 postpartum height, maternal educational status and family socioeconomic status (IWI).  
188 Model 3, which was considered the main model of this study, was additionally adjusted  
189 for child birth weight. To assess whether potential associations observed in model 3 were  
190 mediated by weight at 60 mo, a model 4 was additionally adjusted for weight at the 60-mo  
191 visit. In the analyses of the outcomes systolic and diastolic blood pressure model 4 was  
192 adjusted for height and weight at 60 mo in addition to the adjustments in model 3. The  
193 analyses of estimated birth weight as primary predictor did not include a model 3, and  
194 the model 4 in these analyses was in addition to the adjustments in model 2 adjusted for  
195 child weight at 60 mo. The analyses of weight at 60 mo as primary predictor did not  
196 include a model 4 for all outcomes except for systolic and diastolic blood pressure, where  
197 model 4 was adjusted for height at 60 mo in addition to the adjustments in model 3. To  
198 compare the estimated associations across model 1–4 for a given outcome and exposure, we  
199 used a complete case approach, limiting the analyses to data with complete information on  
200 all covariates in model 4. In addition to the main analyses, we ran a number of sensitivity  
201 analyses. First, information on breastfeeding status was available on a smaller subsample.  
202 Thus, in a sensitivity analysis, we adjusted model 1-4 in all analyses for breastfeeding status  
203 at 4 to 6 mo post-partum to assess the potential change of estimates. Second, we restricted  
204 the analyses to only term average for gestational age children (i.e. children born at term with  
205 at birth weight >2500 g) to assess how the exclusion of small for gestational age children  
206 affected the results. Third, the standard error of the estimated weight gain velocity  
207 trajectories for children contributing with fewer weight measurements from 0-60 mo to the  
208 modelling are expected to be higher than for children with more weight measurement from  
209 0-60 mo. Therefore, in an additional sensitivity analysis, we adjusted for the uncertainty in

210 the estimations of the child-specific weight gain velocities (i.e. estimated standard-errors of  
211 the child-specific random effects). This was done for model 1-4 in all analyses, except for the  
212 analyses where the observed weight at 60 mo was the primary predictor. Lastly, in a final  
213 sensitivity analysis, we accounted all analyses in model 3 (the main model) for multiple  
214 testing using the Benjamini-Hochberg approach (46).

215 All descriptive data are presented as mean (SD) or median (interquartile range) for  
216 continuous variables and percentages for categorical variables. Outcome variables found not  
217 to follow a normal distribution (i.e. insulin, C-peptide, HOMA-IR and triglycerides) were  
218 log-transformed prior to the regression analyses. The resulting effect estimates were back-  
219 transformed and presented as percentwise change. P values <0.05 were considered  
220 statistically significant. The LSME modelling was carried out using the “lmer” function in the  
221 “lme4” package in R. All analyses were carried out in R version 3.4.1 (The R foundation for  
222 Statistical Computing).

## 223 Results

224 A flow diagram of the study participants is shown in **Figure 1**. A total of 375 children were  
225 included in the LSME modelling of weight gain from birth to 60 mo (step 1), and 367 children  
226 had full covariate information for the subsequent regression analyses (step 2). A description  
227 of the study population is shown in **Table 1**. At birth, 10% were low birth weight, and  
228 according to international growth standards (WHO) (47), at 60 mo, the children were on  
229 average slightly thinner, lighter and shorter, and 7% were overweight or obese. The  
230 prevalence of stunting recorded in this population was similar to that of the capital Addis  
231 Ababa (15%), but lower than the national urban average of 25% (48). The average  
232 socioeconomic status of the study population (IWI score: 46 out of 100) was considerably  
233 higher than rural Ethiopia (12 out of 100), and slightly less than the urban areas of the  
234 country (52 out of 100) (41, 42). Boys and girls were equally distributed in the study sample,  
235 and at birth boys had slightly more FFM than girls. The average age at the 60-mo visit was  
236 59.95 (SDs 1.47) mo. For the subsequent regression analyses (step 2), the total number of  
237 children included in the analyses differed depending on the specific outcome, as some  
238 participants had refused the blood sampling or had only been able to deliver an amount of  
239 blood that was insufficient for all biomarkers to be analyzed (**Table 2**).

240

### 241 Child growth from 0-60 mo

242 The children had their weight measured a median (interquartile range) of 9 (8-10) times  
243 from birth to 60 mo and contributed with a total of 3336 weight measurements to the  
244 growth modelling. The distribution of the weight measurements at each follow-up visit is

245 shown in Figure 1. A model with knot points placed at 3, 6, 24 and 48 mo yielded the lowest  
246 Bayesian information criterion value and was selected as the best fitting model. Thus, the  
247 model estimated 6 parameters representing the estimated individual birth weight and 5  
248 weight gain velocities from birth to 60 mo, and thus the deviance from the average birth  
249 weight and weight gain velocities in the periods 0-3, 3-6, 6-24, 24-48 and 48-60 mo. On  
250 average, the weight gain velocities declined gradually over the 5 growth periods from 1,012  
251 g/mo from 0-3 mo to 139 g/mo from 48-60 mo (**Table 3**). The variation in the weight gain  
252 velocities also declined steadily from 0-3 mo to 24-60 mo (**Supplemental Figure 1**).

253 Compared to the international growth standards (WHO), boys and girls were on average  
254 more than 200 g lighter at birth but gained weight at a higher velocity from 0-6 mo.  
255 However, from 6 to 60 mo they gained weight at a lower velocity, resulting in a considerable  
256 average weight deficit of 1,915 g for girls and 1,885 g for boys at 60 mo compared with the  
257 WHO reference. The median growth velocity curve for the study population estimated from  
258 LSME modelling compared with the weight-for-age growth reference is shown in **Figure 2**.

259 Furthermore, the model fits for 3 example children with different growth trajectories is  
260 shown in **Supplemental Figure 2** and the individual model fits for all included children are  
261 shown in **Supplemental Figure 3**. Correlations of varying strengths were observed between  
262 the estimated birth weight and weight gain velocities (**Supplemental Figure 4**). A matrix of  
263 the model assumptions tests of the LSME modelling is shown in **Supplemental Figure 5**.



## 264 Birth weight, child growth and cardiometabolic markers at age 5

265 Associations of estimated birth weight, weight gain velocities and observed weight at 60 mo  
266 with BC and cardiometabolic markers are presented in **Figure 3** and **Supplemental Table 1**.

267 Independent of birth weight and the other covariates (model 3), higher weight accretion  
268 from 48-60 mo was associated with higher levels of insulin, C-peptide, HOMA-IR and HDL  
269 cholesterol at 60-mo. For instance, 1 SDs higher (1 SDs = 50 g/mo) weight accretion from 48-  
270 60 mo was associated with a higher HOMA-IR of 24.3% (95% confidence interval (CI): 9.6,  
271 41.0), insulin of 23.3% (9.6, 38.8), C-peptide of 11.4% (2.7, 20.8) and HDL cholesterol of 0.03  
272 mmol/L (0.00, 0.06). The associations for insulin, C-peptide and HOMA-IR remained after  
273 adjusting for current weight (model 4). Additionally, higher weight at 60 mo was associated  
274 with higher insulin and HOMA-IR. Higher weight accretion from 24-48 mo (1 SDs = 38 g/mo)  
275 was associated with lower levels of blood glucose levels at 60 mo [ $\beta_{\text{model 3}} = -0.11$  mmol/L  
276 (95% CI: -0.21, -0.02)]. Higher weight accretion from 0-3 mo (1 SDs = 165 g/mo) was  
277 associated with higher levels of LDL cholesterol [ $\beta_{\text{model 3}} = 0.08$  mmol/L (0.01, 0.16)]. The  
278 associations for blood glucose and LDL cholesterol remained after adjusting for current  
279 weight. Additionally, in model 4, higher weight accretion from 6-24 mo (1 SDs = 49 g/mo)  
280 was associated with lower levels of LDL cholesterol [ $\beta_{\text{model 4}} = -0.09$  mmol/L (-0.16, -0.01)].

281 We found no associations of weight at birth, weight at 60 mo and weight accretion in any of  
282 the age periods with HbA1c, total cholesterol and triglycerides. Regarding blood pressure at  
283 60 mo, in model 3, positive associations were seen with weight at 60 mo and weight  
284 accretion in the age periods from 0-60 mo (systolic) and 24-60 mo (diastolic). For instance, 1  
285 SDs higher (1 SDs = 2,161 g) weight at 60 mo was associated with a higher systolic blood  
286 pressure of 2.5 mmHg (1.6, 3.3). The positive associations seen for systolic and diastolic

287 blood pressure and weight accretion in the age periods from 0-60 mo disappeared when  
288 adjusting for current weight and height at 60 mo (model 4). However, in model 4, the  
289 positive association of weight 60 mo with systolic blood pressure remained, while negative  
290 association of weight at birth with systolic blood pressure appeared. Birth weight, weight  
291 accretion in the age periods from 0-60 mo and weight at 60 mo were positively associated  
292 with height, waist-circumference, FM and FFM at 60 mo. For instance, in model 3, 1 SDs  
293 higher (1 SDs = 50 g/mo) weight accretion from 48-60 mo was associated with a greater  
294 height of 1.7 cm (95% CI: 1.2, 2.1), waist circumference of 1.6 cm (1.3, 2.0), FM of 0.72 kg  
295 (0.59, 0.85) and FFM of 0.70 kg (0.56, 0.85). The associations largely attenuated after  
296 adjusting for current weight (model 4).

297 The additional sensitivity analyses adjusting the analyses for breastfeeding status at 4-6-mo  
298 post-partum (**Supplemental Figure 6**), restricting the analyses to average for gestational age  
299 children (**Supplemental Figure 7**) and adjusting the analyses for uncertainty of the child-  
300 specific weight gain velocity estimations (**Supplemental Figure 8**) did not alter the presented  
301 associations noticeably. Furthermore, when accounting for multiple testing of the results  
302 presented in model 3 (the main model), the associations of weight accretion from 48-60 mo  
303 with insulin, C-peptide, HOMA-IR and blood pressure as well as most of the other  
304 associations for blood pressure and all associations for height, waist-circumference, FM and  
305 FFM remained significant despite the lower alpha-level in the Benjamini-Hochberg approach  
306 (**Supplemental Figure 9**). However, the associations for blood glucose, HDL- and LDL  
307 cholesterol lost significance when accounting for multiple testing.

## 308 Discussion

309 In this study, we present the first results from a low-income country on the effects of weight  
310 gain velocity in a number of critical windows in early life on linear growth, FFM and markers  
311 of adiposity and cardiometabolic risk in childhood. Independent of birth weight and the  
312 included covariates, weight gain from 48-60 mo was positively associated with insulin, C-  
313 peptide, HOMA-IR, HDL cholesterol, blood pressure, height, waist circumference, FM and  
314 FFM at 60 mo. Moreover, weight gain from 0-3 mo was positively associated with LDL  
315 cholesterol, systolic blood pressure, height, waist circumference, FM and FFM at 60 mo,  
316 while weight gain from 24-48 mo was inversely associated with blood glucose. Birth weight,  
317 on the other hand, was only positively associated with height, waist circumference, FM and  
318 FFM at 60 mo.

319 Few studies from middle- and high-income countries have investigated the relative  
320 importance of birth weight and successive periods of growth in early life on markers of  
321 cardiometabolic risk in early childhood, and evidence from sub-Saharan Africa is limited to  
322 South African children. Crowther et al. showed that higher weight gain from birth to 4 years  
323 and beyond, but not between birth and 1 year, was associated with higher insulin levels in  
324 South African children (13). In a study of Chilean children, Corvalán et al. found that BMI  
325 growth from 1.5-4 years presented the strongest association with a composite metabolic risk  
326 score, but did not find growth from birth to 4 years to be associated with HOMA-IR or  
327 plasma lipids (18). Joglekar et al., on the other hand, found that higher weight gain in  
328 selected periods after 6 mo of age, was associated with systolic blood pressure and HOMA-IR  
329 at 6 years in children from rural India, and that associations were stronger for the most  
330 recent growth periods (11). Similarly, in 6-year-old Dutch children, Voerman et al. found that

331 conditional weight gain from 4-6 years were more strongly associated with insulin and C-  
332 peptide levels at 6 years than earlier periods of weight gain (15). These findings are  
333 consistent with the present study in suggesting that growth in early childhood rather than in  
334 infancy and fetal life is the strongest correlate of levels of C-peptide, insulin and insulin  
335 resistance (HOMA-IR). However, it is difficult to say if increased levels of the studied  
336 cardiometabolic markers are harmful for the children in the longer term or rather represent  
337 normal and beneficial metabolic adaptations to increased weight gain.

338 Birth weight and weight gain velocity in all age periods as well as weight at 60 mo was  
339 positively associated with height, waist circumference, FM and FFM. The associations were  
340 strongest for weight at 60 mo and the most recent growth periods showed larger effect  
341 estimates. Interestingly, the effect sizes for FM were very similar to those of FFM despite  
342 FFM at 60 mo on average was an almost 3 times larger body compartment than FM. In  
343 Indian children, Joglekar et al. found that weight gain from birth to 6 years was associated  
344 with both FM and FFM, but with stronger associations for FFM (11). In Dutch children, de  
345 Beer et al. found that weight gain from 0-12 mo showed a stronger association with FM  
346 compared to FFM at 5-6 years (22). Our analyses suggest that weight gain from birth to 60  
347 mo, is being allocated in relatively larger amounts to FM storage compared to FFM,  
348 irrespectively of weight status at birth. Compared to BC references derived in UK children,  
349 our participants had higher FM (boys: 3.11 vs. 4.19 kg, and girls: 3.97 vs. 4.14 kg) and  
350 markedly lower FFM (boys: 16.35 vs. 12.27 kg, and girls: 14.60 vs. 12.04 kg) at 60 mo (49).  
351 Thus, despite being born with an average deficit in weight that persisted at 60 mo of age  
352 when comparing to the international growth standards (WHO), these children underwent a  
353 significant catch-up in FM associated with weight changes in both infancy and early

354 childhood and resulting in a considerably higher FM at 60 mo compared to children from a  
355 high-income country.

356 The mechanisms that explain the associations of variability in early growth with markers of  
357 adiposity and cardiometabolic risk in childhood remains unclear, but they may include  
358 alterations to the microbiome, hepatic endoplasmic reticulum stress and MicroRNAs  
359 associated with impaired insulin signaling, as well as epigenetic and transcriptional  
360 mechanisms (50). The epigenetic mechanisms include DNA methylation and histone  
361 modifications that induce changes in the structure and function of vital organs related the  
362 metabolic homeostasis such as the liver, adipose tissue and pancreas (50-52). Ibáñez et al.  
363 found that catch-up in weight from birth to 4 years in children small-for-gestational age  
364 elevated adiposity and promoted insulin resistance (14). Moreover, as proposed in the  
365 capacity-load model by Wells, metabolic-load (e.g. accumulated FM) may challenge  
366 metabolic homeostasis (e.g. promote insulin resistance) if it is not resolved by the metabolic  
367 capacity for instance through pancreatic insulin production (53). Thus, viewing our findings  
368 through the lens of the capacity-load model, it is possible that accelerated weight gain has  
369 resulted in elevated levels of C-peptide, insulin and HOMA-IR through an elevated metabolic  
370 load (e.g. accumulated FM). Ethiopia and most other Sub-Saharan African countries are  
371 currently undergoing a rapid nutritional transition with increased energy availability from  
372 the diet (26, 54). Thus, our findings that childhood growth rather than fetal and infant  
373 growth was associated with markers of glucose metabolism may reflect an increased  
374 exposure to an obesogenic environment, where the excess energy available from the diet is  
375 being allocated to weight gain in the form of adipose tissue storage (16), which results in  
376 higher levels of circulating insulin and C-peptide. However, as insulin is a peptide hormone

377 associated with growth stimulation and fat accumulation, we cannot rule out that the  
378 associations discussed above is a result of reverse causation (55). Furthermore, it is possible  
379 that associations of fetal and infant weight gain with markers of glucose metabolism will  
380 appear at later ages.

381 When adjusting for current weight at 60 mo in model 4 to assess any potential mediation  
382 through current weight, the associations of weight gain with blood glucose, C-peptide,  
383 insulin, HOMA-IR and LDL cholesterol persisted, but the positive associations with blood  
384 pressure disappeared. Interestingly, a new inverse association between birth weight and  
385 systolic blood pressure appeared. Correspondingly, studies from both rural and urban India  
386 have found that an inverse association between birth weight and systolic blood pressure  
387 appeared only when adjusting for current weight (11, 12). This finding may reflect the  
388 positive association of birth weight with nephron number (56), an important component of  
389 metabolic capacity (57), and the strong association of weight at 60 mo with accumulated FM.  
390 Thus, holding constant for weight at 60 mo, those with larger birth weight and more  
391 nephrons have lower blood pressure. The positive associations of weight at birth and weight  
392 gain in all growth periods with FM and FFM at 60 mo lost significance after adjusting for  
393 current weight at 60 mo. However, this was expected due to the close relationship of weight  
394 with FM and FFM, and similar results have been seen in comparable studies from high  
395 income countries (20, 58).

396 It should be acknowledged, that none of the children had clinically pertinent levels of any of  
397 the cardiometabolic markers, but as cardiometabolic risk markers have been shown to track  
398 from childhood to adulthood (59, 60) and adiposity in childhood increase the risk of obesity  
399 and cardiometabolic disease in adulthood (61-63), further follow-up studies are needed to

400 examine how the observed changes in adiposity and cardiometabolic risk markers are  
401 developing in later childhood and if accelerated growth after 48 mo really is a risk factor for  
402 cardiometabolic risk later in life.

### 403 *Strengths and limitations*

404 The present study has several important strengths. First, the modelling of growth is based on  
405 more than 3300 weight measurements over 12 visits with detailed assessments the first 6  
406 mo of life. Second, the large number of observations and follow-up visits as well as the  
407 repeated measurement data structure enabled us to use LSME modelling to estimate the  
408 non-linear relationship of weight gain in children. LSME modelling is particularly useful for  
409 repeated measures data as it allows for unbalanced and missing data, under an assumption  
410 that data are missing at random, as well as accounts for dependencies in the intra-individual  
411 weight measurements. Thus, it enabled a more optimal use of the available observations, by  
412 allowing participants to be included in the study despite contributing with few weight  
413 measurements. This reduced potential bias by selective dropout and increased the statistical  
414 power of the study significantly. Third, FM and FFM were measured using an air  
415 displacement plethysmograph, which is an accurate, precise and feasible method for  
416 assessment of BC in children (37, 38). Lastly, over the 5-year follow-up period with 12 visits,  
417 it was possible to include 65% of the 571 children included in the follow-up study after birth  
418 in the 5-year follow-up visit. However, we cannot rule out that the observed differences  
419 between included and excluded participants could have caused a slight selection bias in the  
420 estimated associations (**Supplemental Table 2**). However, our study also has limitations.  
421 First, while the LSME modelling can deal with children that have not been measured at all  
422 possible visits by allowing those subjects to assume a growth trajectory closer to the

423 population average, missing visits inevitably makes the estimation of the individual growth  
424 trajectories more uncertain. However, in a sensitivity analysis we adjusted our analyses for  
425 this uncertainty, and it did not make any difference in the estimated associations  
426 (Supplemental Figure 8). Second, for feasibility reasons we standardized the fasting for the  
427 5-year-old children to 3 hours. Not having an overnight fast may have resulted in some non-  
428 differential misclassification of the effect sizes, and thus may have caused the effect  
429 estimates to move towards zero. Finally, we only included healthy mother-child pairs living  
430 in the town of Jimma, which prevents us from generalizing our results to sick or  
431 undernourished children, and children living in more rural parts of Ethiopia or rural parts in  
432 other sub-Saharan African countries. Whether the findings from this study are reproducible  
433 in these populations should be further studied.

## 434 **Conclusion**

435 Novel statistical methods were used to model weight gain velocities from 0-60 mo, to study  
436 the relative importance of weight at birth and weight gain from 0-60 mo with  
437 cardiometabolic markers and BC at 60 mo. Our findings suggest that children with a higher  
438 weight at 60 mo who gained weight at an accelerated rate, particularly after 48 mo of age,  
439 have higher levels of key cardiometabolic markers related to insulin, C-peptide, systolic  
440 blood pressure, height, waist circumference, FM and FFM. Collectively, our analyses  
441 therefore demonstrate that not all growth periods are equally important for the  
442 development of BC and cardiometabolic profile in childhood.



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## **Author contributions**

TG, PK, JCKW, KFM, HF and GSA designed the research; RW, TG, BA, MA, PK, AA, ZG and GSA conducted the research; RW and DV performed the statistical analysis; RW and GSA wrote the paper; All authors revised and approved the final manuscript.

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## Tables

**Table 1** Background characteristics at birth and at 60 mo of urban Ethiopian children and their mothers for the full sample of children included in the growth modelling and attending the 60 mo follow-up visit <sup>1</sup>

|   | Full sample<br>(n = 375) | Girls<br>(n = 188) | Boys<br>(n = 187) | P value <sup>2</sup> | Missing, n |
|---|--------------------------|--------------------|-------------------|----------------------|------------|
| <b>Maternal characteristics</b>                   |                          |                    |                   |                      |            |
| Age at birth (years)                              | 24.6 ± 4.7               | 24.9 ± 4.8         | 24.3 ± 4.7        | 0.241                | 0          |
| Postpartum height (cm)                            | 157.1 ± 6.1              | 157.5 ± 6.2        | 156.7 ± 5.9       | 0.212                | 8          |
| Postpartum body mass index (kg/m <sup>2</sup> )   | 22.23 ± 3.51             | 22.22 ± 3.43       | 22.25 ± 3.61      | 0.947                | 28         |
| Birth order of current child                      |                          |                    |                   | 0.223                | 0          |
| First   | 50.1                     | 46.8               | 53.5              |                      |            |
| Second  | 26.4                     | 26.1               | 26.7              |                      |            |
| Third or above                                    | 23.5                     | 27.1               | 19.8              |                      |            |
| Breast feeding status at 4 to 6 mo post-partum    |                          |                    |                   | 0.991                | 43         |
| Exclusive   | 12.3                     | 12.0               | 12.7              |                      |            |
| Almost exclusive (water given)                    | 21.4                     | 21.0               | 21.8              |                      |            |
| Predominant                                       | 60.2                     | 61.1               | 59.4              |                      |            |
| Partial or none                                   | 6.0                      | 6.0                | 6.1               |                      |            |
| Maternal education                                |                          |                    |                   | 0.111                | 0          |
| No school   | 6.9                      | 5.3                | 8.6               |                      |            |
| Some primary school                               | 44.3                     | 45.2               | 43.3              |                      |            |
| Completed primary school                          | 16.0                     | 20.2               | 11.8              |                      |            |
| Completed secondary school                        | 18.9                     | 16.0               | 21.9              |                      |            |
| Higher education                                  | 13.9                     | 13.3               | 14.4              |                      |            |
| Socioeconomic status (International Wealth Index) | 45.7 ± 17.3              | 46.4 ± 17.3        | 44.9 ± 17.2       | 0.380                | 0          |
| <b>Child characteristics at birth</b>             |                          |                    |                   |                      |            |
| Gestational age (weeks)                           | 39.0 ± 1.0               | 39.1 ± 1.0         | 39.0 ± 1.0        | 0.384                | 0          |
| Weight (kg)                                       | 3.04 ± 0.41              | 3.01 ± 0.41        | 3.07 ± 0.41       | 0.117                | 0          |
| Length (cm)                                       | 49.1 ± 2.0               | 48.9 ± 2.0         | 49.3 ± 2.0        | 0.055                | 0          |
| Fat mass (kg)                                     | 0.22 ± 0.16              | 0.23 ± 0.16        | 0.21 ± 0.17       | 0.106                | 2          |
| Fat-free mass (kg)                                | 2.83 ± 0.32              | 2.77 ± 0.32        | 2.88 ± 0.32       | 0.002                | 2          |
| Low birth weight (%) <sup>3</sup>                 | 9.6                      | 10.6               | 8.6               | 0.611                | 0          |
| <b>Child characteristics at 60 mo</b>             |                          |                    |                   |                      |            |
| Age at 60 mo visit (mo)                           | 59.95 ± 1.47             | 59.89 ± 1.60       | 60.02 ± 1.33      | 0.422                | 0          |
| Weight (kg)                                       | 16.38 ± 2.16             | 16.30 ± 2.22       | 16.45 ± 2.10      | 0.509                | 0          |
| Height (cm)                                       | 104.3 ± 4.5              | 104.2 ± 4.2        | 104.4 ± 4.7       | 0.700                | 0          |
| Weight for age z-score <sup>4</sup>               | -0.86 ± 0.90             | -0.83 ± 0.88       | -0.88 ± 0.92      | 0.613                | 0          |
| Height for age z-score                            | -1.14 ± 0.92             | -1.08 ± 0.86       | -1.20 ± 0.98      | 0.193                | 0          |
| BMI for age z-score                               | -0.22 ± 0.90             | -0.27 ± 0.95       | -0.16 ± 0.85      | 0.270                | 0          |
| Underweight <sup>5</sup>                          | 9.1                      | 8.5                | 9.6               | 0.844                | 0          |
| Stunted <sup>6</sup>                              | 14.9                     | 12.8               | 17.1              | 0.300                | 0          |
| Wasted by BMI (Thinness) <sup>7</sup>             | 2.7                      | 4.3                | 1.1               | 0.105                | 0          |
| Overweight <sup>8</sup>                           | 5.1                      | 5.3                | 4.8               | 1.000                | 0          |
| Obese <sup>9</sup>                                | 1.6                      | 1.6                | 1.6               | 1.000                | 0          |

<sup>1</sup> Values are mean ± SDs for continuous normally distributed variables and percentages for categorical variables. <sup>2</sup> Differences between boys and girls were calculated by One-way ANOVA F-test for continuous variables, Pearson's Chi-Square test of independence for categorical variables with expected counts above 5 in all cells and Fisher's exact test of independence for categorical variables with expected counts in any cell below 5. <sup>3</sup> Low birth weight is defined as birth weight <2500 g. <sup>4</sup> Z-scores are derived using the 2006 (children aged <61 mo) and 2007 (children aged ≥61 mo) World Health Organization (WHO) child growth standards (47). <sup>5</sup> Weight for age more than 2 SDs below the age- and sex-specific median of the WHO child growth standards. <sup>6</sup> Height for age more than 2 SDs below the age- and sex-specific median of the WHO child growth standards. <sup>7</sup> BMI for age more than 2 SDs below the age- and sex-specific median of the WHO child growth standards. <sup>8</sup> BMI-for-age from 1 to 2 SDs above the sex-specific median of the WHO child growth standards. <sup>9</sup> BMI-for-age more than 2 SDs above the sex-specific median of the WHO child growth standards.

**Table 2** Cardiometabolic markers, anthropometry and body composition at 60 mo of age of urban Ethiopian children and their mothers for the full sample of children included in the growth modelling and attending the 60 mo follow-up visit <sup>1</sup>

|   | Full sample<br>(n = 375) | Girls<br>(n = 188) | Boys<br>(n = 187) | P value <sup>2</sup> | Missing, n |
|---|--------------------------|--------------------|-------------------|----------------------|------------|
| <b>Glucose metabolism</b>                             |                          |                    |                   |                      |            |
| Glucose, whole blood (mmol/L)                         | 5.90 ± 0.84              | 5.85 ± 0.76        | 5.96 ± 0.91       | 0.201                | 27         |
| HbA1c, whole blood (mmol/mol)                         | 38 ± 4                   | 38 ± 4             | 38 ± 4            | 0.789                | 92         |
| Insulin, serum (μU/mL) <sup>3</sup>                   | 6.03 (3.25-11.20)        | 7.28 (4.07-12.76)  | 5.42 (3.02-9.34)  | 0.004                | 35         |
| C-peptide, serum (ng/mL) <sup>3</sup>                 | 1.08 (0.68-1.60)         | 1.16 (0.75-1.90)   | 0.94 (0.56-1.45)  | 0.001                | 40         |
| HOMA-IR <sup>3,4</sup>                                | 1.32 (0.67-2.46)         | 1.58 (0.85-2.65)   | 1.12 (0.57-2.09)  | 0.007                | 35         |
| <b>Lipids</b>   |                          |                    |                   |                      |            |
| Total cholesterol, serum (mmol/L)                     | 3.43 ± 0.61              | 3.47 ± 0.64        | 3.38 ± 0.58       | 0.165                | 31         |
| LDL cholesterol, serum (mmol/L)                       | 1.66 ± 0.56              | 1.71 ± 0.56        | 1.61 ± 0.55       | 0.097                | 32         |
| HDL cholesterol, serum (mmol/L)                       | 0.80 ± 0.26              | 0.79 ± 0.28        | 0.80 ± 0.24       | 0.711                | 36         |
| Triglycerides, serum (mmol/L) <sup>3</sup>            | 0.96 (0.74-1.29)         | 0.95 (0.76-1.31)   | 0.97 (0.72-1.28)  | 0.605                | 36         |
| <b>Blood pressure</b>                                 |                          |                    |                   |                      |            |
| Systolic (mmHg)                                       | 87.9 ± 7.3               | 88.1 ± 7.1         | 87.7 ± 7.5        | 0.577                | 2          |
| Diastolic (mmHg)                                      | 54.4 ± 8.5               | 54.7 ± 8.5         | 54.1 ± 8.4        | 0.472                | 2          |
| <b>Anthropometry and body composition</b>             |                          |                    |                   |                      |            |
| Body mass index (kg/m <sup>2</sup> )                  | 15.02 ± 1.29             | 14.98 ± 1.44       | 15.06 ± 1.13      | 0.570                | 0          |
| Waist circumference (cm)                              | 51.6 ± 3.2               | 51.4 ± 3.4         | 51.8 ± 2.9        | 0.314                | 1          |
| Fat mass (kg)   | 4.19 ± 1.32              | 4.19 ± 1.46        | 4.19 ± 1.16       | 0.991                | 18         |
| Fat-free mass (kg)                                    | 12.20 ± 1.45             | 12.10 ± 1.36       | 12.29 ± 1.54      | 0.216                | 18         |
| Fat mass index (kg/m <sup>2</sup> ) <sup>5</sup>      | 3.84 ± 1.12              | 3.84 ± 1.23        | 3.83 ± 0.98       | 0.916                | 18         |
| Fat-free mass index (kg/m <sup>2</sup> ) <sup>5</sup> | 11.20 ± 0.88             | 11.15 ± 0.91       | 11.26 ± 0.84      | 0.261                | 18         |

<sup>1</sup> Values are mean ± SDs for continuous variables that are normally distributed and median (interquartile range) for continuous variables that are not following a normal distribution. <sup>2</sup> Differences between boys and girls were calculated by One-way ANOVA F-test for continuous normally distributed variables. Variables found not to follow a normal distribution were log transformed prior to the tests of group differences. <sup>3</sup> Nonnormally distributed. <sup>4</sup> Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as insulin (μU/mL) × glucose (mmol/l) / 22.5. <sup>5</sup> Fat mass index and Fat-free mass index were calculated by dividing FM and FFM with the squared height in meter, respectively.

**Table 3** Average estimated weight at birth, weight gain velocities from birth to 60 mo estimated with linear-spline mixed effects modelling and observed weight at the 60 mo visit in Ethiopian urban children <sup>1</sup>

|   | Full sample<br>(n = 375) | Girls<br>(n = 188) | Boys<br>(n = 187) | WHO growth standards <sup>2</sup> |        |
|---|--------------------------|--------------------|-------------------|-----------------------------------|--------|
|   |                          |                    |                   | Girls                             | Boys   |
| Weight (g)<br>Birth visit               | 3,048 ± 293              | 3,008 ± 282        | 3,089 ± 300       | 3,232                             | 3,346  |
| Weight gain velocity (g/mo)<br>0-3 mo   | 1,012 ± 165              | 984 ± 153          | 1,041 ± 173       | 871                               | 1,010  |
| Weight gain velocity (g/mo)<br>3-6 mo   | 516 ± 148                | 506 ± 153          | 527 ± 143         | 484                               | 519    |
| Weight gain velocity (g/mo)<br>6-24 mo  | 206 ± 49                 | 206 ± 50           | 206 ± 48          | 232                               | 234    |
| Weight gain velocity (g/mo)<br>24-48 mo | 140 ± 38                 | 141 ± 38           | 140 ± 37          | 191                               | 175    |
| Weight gain velocity (g/mo)<br>48-60 mo | 139 ± 50                 | 143 ± 58           | 134 ± 40          | 179                               | 166    |
| Observed weight (g)<br>60 mo visit      | 16,378 ± 2,161           | 16,304 ± 2,223     | 16,452 ± 2,100    | 18,219                            | 18,337 |

<sup>1</sup> Values are mean ± SDs. <sup>2</sup> Weight gain velocities (g/mo) were calculated by the difference in the estimated median weight in g at the end of the age interval and at the beginning of the age interval divided by the length in mo of the age interval using the World Health Organization 2006 growth standards (47).

## Legends for figures

**Figure 1.** Flow diagram of the study participants and number of children at each follow-up visit from birth to 60 mo of age.

**Figure 2.** The median weight gain velocity curve for the study population estimated from linear spline mixed effects modelling. The dashed lines show the 95% prediction interval. The vertical grey lines show the selected knot points, and the slopes between the knot points express the estimated median weight gain velocity in each of the age intervals. The shaded grey areas show the reference in standard deviation scores from the median weight-for-age according to the World Health Organization international growth standards.

**Figure 3.** Associations of estimated birth weight, weight gain velocities and observed weight at 60 mo with height, fat-free mass and markers of adiposity (A) and cardiometabolic risk (B). The coefficients (and 95% CI) displayed on the forest plot were derived from separate multiple linear regression models and represent the change in the 60-mo outcomes per study population standard deviation score increase in estimated birth weight, weight gain velocities and observed weight at the 60-mo visit, respectively. The linear spline mixed effects model used to estimate birth weight and weight gain velocities had 4 internal knot points at 3, 6, 24 and 48 mo, yielding the 5 growth periods 0-3, 3-6, 6-24, 24-48 and 48-60 mo. Weight at 60 mo was standardized prior to the analyses. Nonnormally distributed variables (i.e. insulin, C-peptide, HOMA-IR and triglycerides) were log transformed prior to the regression analysis. The resulting effect

estimates were back-transformed and presented as percentwise change. Model 1 was adjusted for sex, birth order and gestational age. Model 2 was additionally adjusted for the child's exact age at the 60-mo visit, maternal age at delivery, maternal postpartum height, maternal educational status and family socioeconomic status (International Wealth Index). Model 3 was additionally adjusted for child birth weight. Model 4 was additionally adjusted for child weight at 60 mo. In the analyses of the outcomes systolic and diastolic blood pressure model 4 was adjusted for height and weight at 60 mo in addition to the adjustments in model 3. The analyses of estimated birth weight as primary predictor did not include a model 3 and model 4, in these analyses, was in addition to the adjustments in model 2 adjusted for child weight at 60 mo. The analyses of weight at 60 mo as primary predictor did not include a model 4 for all outcomes except for systolic and diastolic blood pressure, where model 4 was adjusted for height at 60 mo in addition to the adjustments in model 3. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



Fig 1

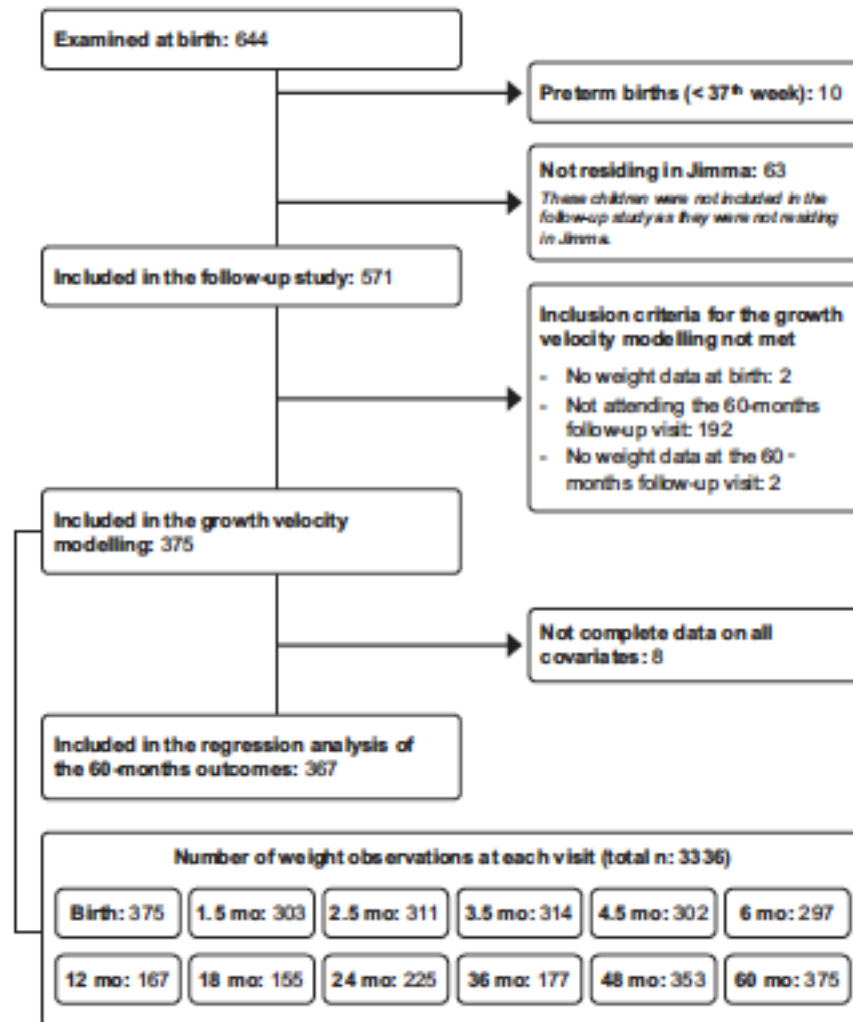


Fig 2

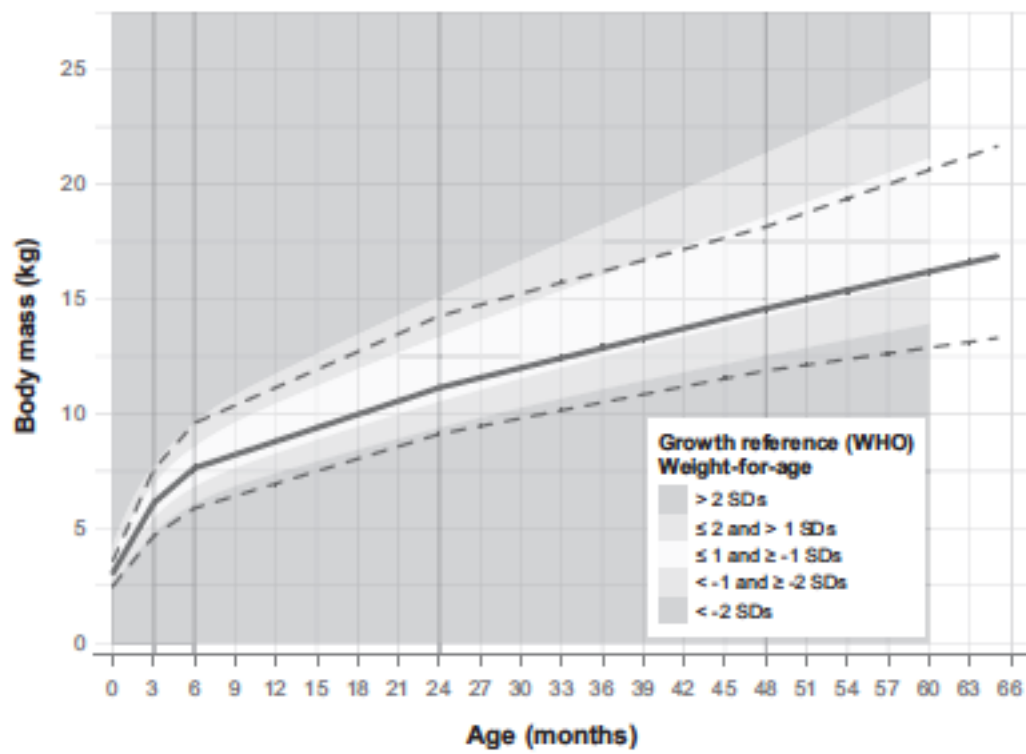


Fig 3

